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STEM CELLS IN INFLAMMATION AND REGENERATION: FOCUSING ON ANIMAL MODELS OF MULTIPLE SCLEROSIS AND SPINAL CORD INJURY

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Karolinska Institutet

Stockholm 2018

Published by Karolinska Institutet.
Printed by Eprint AB 2018

ISBN 978-91-7831-263-4

On the cover: Represents the work presented in this thesis. Front cover, rat spinal cord with NeuN (greenish yellow) showing neurons in the spinal cord and back cover rat brain showing GFAP astrocyte (green) and Sox2 (red) neural stem cells hidden in between astrocytes. Neural stem cells originated from astrocytes were studied during spinal cord inflammation

Stem cells in inflammation and regeneration: focusing on animal models of multiple sclerosis and spinal cord injury

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my lovely family

ABSTRACT

The main objective of regenerative medicine is to replace or restore injured cells and tissues in the body. Stem cells are identified playing a key role in the regeneration but action of inflammatory mediators in disease is not well understood. In this work, stem cells from bone marrow, adult brain and spinal cord were studied with regards to regenerative possibilities.

Paper I, Cell fusion has been observed during development and adult regeneration processes such as in heart, muscle and liver. Scientists have reported BMDC fusion with Purkinje neurons in cerebellum and that the BMDC nucleus can be reprogrammed to express Purkinje neuronal genes. Here we described in finding that, cell fusion between bone marrow derived cells and motor neurons in the spinal cord can take place. This is the first report demonstrating that motor neurons in spinal cord are able to fuse with hematopoietic cells during inflammation. We also identified the fusion phenomenon in spinal interneurons and in the olfactory bulb. In order to identify fusion event outside cerebellum, we used mice bone marrow transplantations and the EAE animal model. We identified fused motor neurons in the ventral horn expressing NeuN,and ChAT. Motor neuron identity was confirmed by tracing with axons in the sciatic nerve fibers to the cell body location in the spinal cord. We also observed that these fused neurons often are bi-nucleated. Yet, not all fused motor neurons were bi-nucleated, this might be due to technical difficulties or that other mechanisms might playing a role during fusion.

Paper II, Is focused on how inflammation affects endogenous neural stem cells distant from EAE lesions in spinal cord. We isolated NSC from different levels of the EAE affected spinal cord and we report that inflammation during EAE can affect NSC that are distant from lesion site. NSC from normal appearing spinal cord showed increased proliferation, altered gene expression and differentiation profile *in-vitro*. We detected that, NSC in normal appearing spinal cord displayed increased neurogenesis and reduced oligodendrocyte differentiation after the inflammatory event.

P**aper III**, We asked whether transplantation of NSC from subventricular zone improves hind limb function in spinal cord injured rats. For this, we isolated SVZ-NSC expressing eGFP and transplanted into immune compatible rats after SCI. We observed that transplanted NSC survived until 12weeks of post injury, filled the cyst and differentiated predominantly into oligodendrocytes (CC1), astrocytes (GFAP) and few neurons (ß-III tubulin). We observed that the animals received NSC improved hind limb function, decreased pro-inflammatory profile in cerebrospinal fluid and altered gene expression in the grafted cells. Further, ablation of the transplanted NSC using diphtheria receptor transfection, confirmed that, recovery of animal was due to the influence of the transplanted NSC.

Conclusion: BMDC fuses with motor neuron and interneurons in entire neuroaxis and these events increases during inflammation. Inflammatory lesions can affect differentiation and proliferation of NSC that are present in the normal appearing spinal cord distant from the site of inflammation. Finally, transplantation of NSC after spinal cord injury improves hindlimb recovery in rats.

Key words: neural stem cells, inflammation, cell fusion, regeneration, reprogramming, EAE, spinal cord injury, transplantations

LIST OF SCIENTIFIC PAPERS

I. **Sankavaram SR**, Svensson MA, Olsson T, Brundin L, Johansson CB. *Cell Fusion along the Anterior-Posterior Neuroaxis in Mice with Experimental Autoimmune Encephalomyelitis*. **PLoS One**. **2015** Jul 24;10(7):0133903

II. Arvidsson L, Covacu R, Estrada CP, **Sankavaram SR**, Svensson M, Brundin L. *Long-distance effects of inflammation on differentiation of adult spinal cord neural stem/progenitor cells*. **JNeuroimmunol**.**2015**Nov15;288:47-55.

III. **Sankavaram SR**, Ramil Hakim, Arvid Frostell, Ruxandra Covacu, Susanne Neumann, Mikael Svensson, Lou Brundin. *Analysis of possible mechanisms behind functional recovery following neural progenitor cell transplantation into spinal cord injury*. **Manuscript** *(submitted)*

ADDITIONAL CONTRIBUTIONS

I. Pérez Estrada C, Covacu R, **Sankavaram SR**, Svensson M, Brundin L. *Oxidative stress increases neurogenesis and oligodendrogenesis in adult neural progenitor cells*. ***Stem CellsDev*. 2014** Oct 1;23(19):2311-27

II. Ramil Hakim, Ruxandra Covacu, Vasilios Zachariadis, **Sreenivasa Sankavaram** Arvid Frostell, Lou Brundin, Mikael Svensson. *Syngeneic, in contrast to Allogeneic, Mesenchymal Stem Cells have Superior Therapeutic Potential Following Spinal Cord Injury*. **Manuscript** *(Submitted)*

CONTENTS

LIST OF ABBREVIATIONS

NSC	Neural Stem Cell
BBB	Blood Brain Barrier
CNS	Central Nervous System
PNS	Peripheral Nervous System
MAI	Myelin-Associated Inhibitor
FGF	Fibroblast Growth Factor
EGF	Epidermal Growth Factor
RAG	Regeneration Associated Gene
CNTF	Ciliary Neurotrophic Factor
NGF	Nerve Growth Factor
BDNF	Brain Derived Growth Factor
SCNT	Somatic Cell Nuclear Transfer
BMDC	Bone Marrow Derived Cell
iPS	Induced Pluripotent Stem Cell
EAE	Experimental Autoimmune Encephalomyelitis
SVZ	Sub Ventricular Zone
SGZ	Sub Granular Zone
GFAP	Glial Fibrillary Acidic Protein
SCI	Spinal Cord Injury
TBI	Traumatic Brain Injury
DTR	Diphtheria Toxin Receptor
AFF-1	Anchor cell Fusion Failure-1
DT	Diphtheria Toxin
ESC	Embryonic Stem Cells
RMS	Rostral Migratory Stream
CSF	Cerebro Spinal Fluid
NAWM	Normal Appearing White Matter
IL-1	InterLeukin-1
PDGF	Platelet Derived Growth Factor
CSPG	Chondroitin Sulfate ProteoGlycan

1 INTRODUCTION

1.1 REGENERATION IN CENTRAL NERVOUS SYSTEM

During early 20th century Ramon Y Cajal who was a pioneer in the neuroscience field with his structural illustrations of the brain introduced the notion that adult brain neurons can't be regenerated after termination of development. He also reported that the brain neurons have fixed connections that would disappear during aging [1-3]. This neuron theory prevailed due to available resources 100 years back and dominated in the field of neuroscience for long time, and the concept became the foundation of modern neuroscience. However, this dogma was challenged by many studies that provided clear experimental evidence on the existence of adult neurogenesis. In the first contradictory experiment in 1963 Joseph Altman injected ^{3}H-thymidin in adult rats and cats intraperitonially and observed thymidine incorporation in the neurons[4]. Later experiments provided ample evidence on adult neurogenesis in birds[5], rodents[6] and non-human primates[7]. In 1999 Alvarez-Buylla *et al*., showed that astrocytes that reside in the subventricular zone are the neural stem cells and generate new neurons in olfactory bulb[8]. In the same year, Johansson *et.al*., demonstrated the presence of ependymal stem cells in the spinal cord with neural stem cell properties and that they proliferate, migrate and contribute to scar in spinal cord injury[9]. Later Barnabe-Heider *et al*., showed in transgenic mice that, the presence of different population of stem cells in intact spinal cord and after spinal cord injury a large number of cells were generated from self-duplication of ependymal cells and astrocytes[10]. This was the first evidence with genetic fate mapping indicating that stem cells in intact spinal cord acts differently in injury than in normal homeostasis. Fagerlund *et al*., identified neural stem cells in brainstem, they demonstrated that during hypoglossal nerve avulsion injury, ependymal cells in brainstem proliferate, migrated but rarely differentiated into neurons[11]. All these experiments led to the conclusion that the adult mammalian brain neurogenesis takes place in two regions in normal homeostasis; the subventricular zone (SVZ) of lateral ventricles and subgranular zone of dentate gyrus in hippocampus[12]. While, upon injury, ependymal cells acts as stem cells in spinal cord[13] and brainstem in rodents[11].

The first report in human hippocampal adult neurogenesis came from Eriksson *et al*. He identified BrdU positive neurons in human hippocampus from patients that had undergone BrdU treatment for cancer, indicating newly generated neuron in adult humans[14]. Later, these results were confirmed in different laboratories[15]. In another attempt to identify neurogenesis in humans, Spalding et.al., used C^{14} dating method to determine the timing of birth of individual cells thereby identify neurogenesis[16]. Using this method Frisen *et.al*., showed neurogenesis in human olfactory bulb, but it declined in adults as compared to children[17]; in human striatum[18]. Using the C^{14} approach Frisen *et al*., showed that human neurogenesis occurs after stroke, but these neurons were unable to integrate with the host tissue[19]. Later it was confirmed in animal models that astrocytes in striatum generate new neurons after stroke[20]. Now it's been widely accepted that neurogenesis and regeneration can occur in the mammalian central nervous system (CNS). However, recently, two independent reports on adult human neurogenesis reignited the debate; Sorrells et.al., 2018 concludes that neurogenesis in adult human

hippocampal dentate gyrus is at undetectable level where as Boldrini *et al*., 2018 concludes the opposite i.e., lifelong neurogenesis in human hippocampus[21,22].

1.2 CNS REGENERATION ACROSS THE SPECIES

Tissue regeneration and repair processes occurs in almost all organisms but when we observe closely in animal kingdom, the degree of regeneration vary greatly between species. In primitive animals like invertebrates to higher order mammals, wound healing process is conserved whereas regeneration potentials of CNS greatly diminished. This might be due to that the specific genes expressed in regenerative species were lost/silenced during evolution. Many evolutionary conserved genes have been identified across the animal kingdom, such as *Caenorhabditis elegans* genes (Dural lucine zipper Kinase-1 DLK-1 in axon regeneration are conserved in human[23]) in human development and disease. Therefore, understanding the genetic mechanisms across species might be useful in studies of CNS regeneration. I would like to discuss briefly about how regeneration event has been changed during millions of years of evolution from invertebrates to mammals.

Invertebrates like planarian and Hydra regenerates not only CNS but the entire organism. The planaria ability to regrow the entire body depends on the presence of number of neoblasts. Neoblast is a specialized adult cell that is pluripotent and constitutes at about 25-30% of the cell population. These neoblast actively divides, migrates and accumulates as groups of cells at the vicinity of the injury called blastema. Neoblasts in Planaria are able to generate all the cells of the body and are pluripotent (can be compared with pluripotent embryonic stem cells in mammals). These neoblasts in blastema divide and differentiate into sub-populations called clonogenic neoblasts[24]. These clonogenic neoblasts are lineage-restricted (similar to multipotent adult stem cells in mammals), and these further differentiates into adult tissue and can regenerate the entire animal[25]. Recently, using single cells RNA sequencing technique, a sub-population of neoblasts called nb2 cells were identified and also transplantation of single pluripotent nb2 cell regenerated entire Planaria[26] Unlike Planaria, the Hydra doesn't have pluripotent stem cells but it has three different types of stem cells in its body (ectodermal stem cells, endodermal epithelial stem cells and intestinal stem cells) and these three types of stem cells together contribute total body regeneration[27]. Primitive vertebrates such as amphibians and fish have tremendous potential to regenerate almost the entire CNS, telencephalon, preoptic region of hypothalamus, midbrain, cerebellum and the spinal cord[28-32] but fail to regenerate an entire body. Urodeles amphibians such as salamander is well studied in regeneration experiments and has a remarkable capacity to regenerate a wide range of tissues and organs after injury/amputation including limbs[33,34], tails[35], jaws[33], heart, spinal cords[35], mid brain[36], retina and the eye[37]. Several mechanisms by which salamander limb regeneration can occur was identified including: the presence of lineage restricted progenitors[38], dedifferentiation of Pax7 cells[39,40] and activation of muscle satellite cells[33,39]. Zebrafish (teleost fish) can regrow heart, fins, retina, spinal cord upon amputation by different mechanisms.

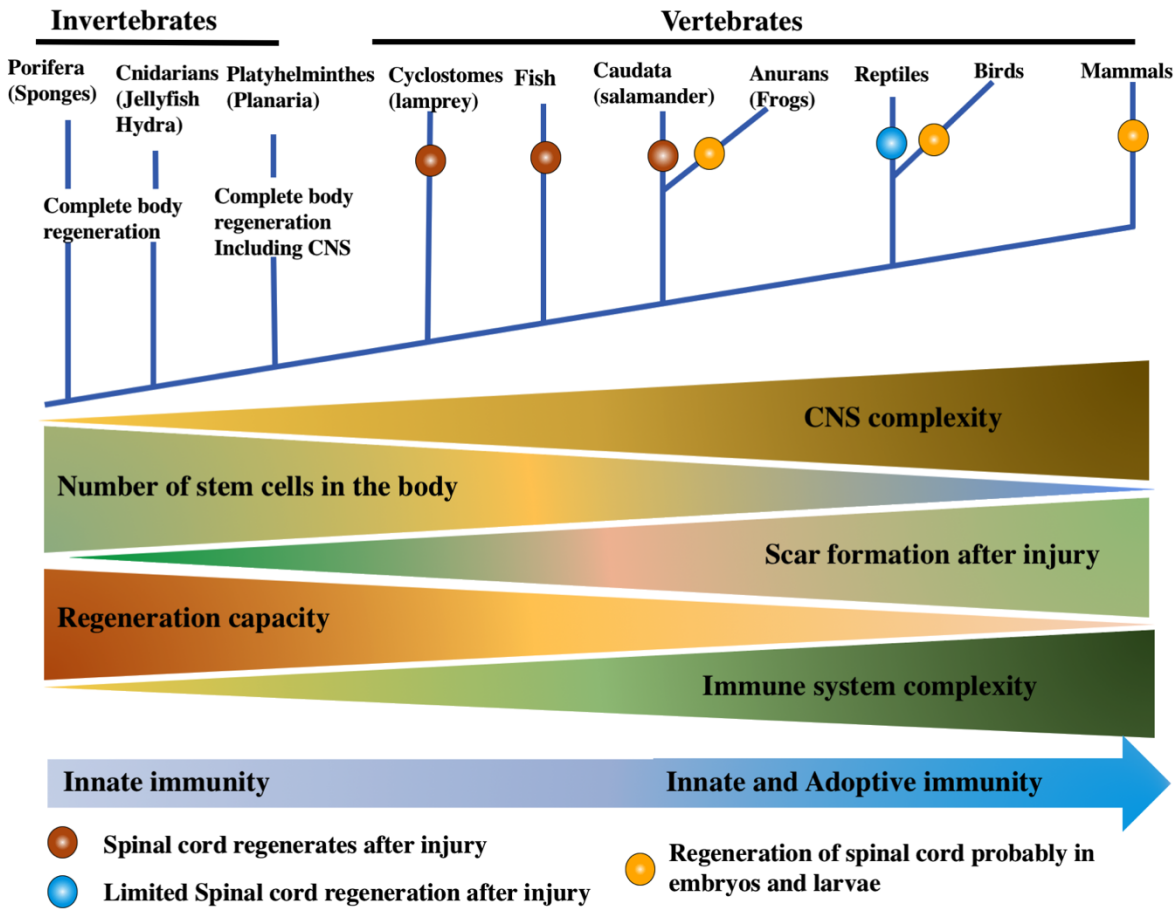

Figure 1. Illustration representing how regeneration capacity compromised during evolution:

Invertebrates have less complex CNS and immune system than in the mammals. At about 20-30% of body cells acts as pluripotent stem cells (Planaria) whereas in mammals limited multipotent stem cells identified in specialized niches (Modified and reproduced with permission from publishers Grandel *et al* 2013[44], Popvich *et al*., 2008[45] Andong Z *et al*. 2016[46], Tanaka E *et al* 2009[47])

These mechanisms identified in Zebrafish robust regeneration are; lineage-restricted progenitors, which migrate into amputation plane and forms blastema in similar way as in salamanders[41,42], dedifferentiation of osteoblast that forms bone and activation of stem cells, which regenerate muscle tissue[43]. Neurogenesis in birds was first discovered in songbirds[5] in which during the spring male birds produce songs to attract female birds whereas in fall they were not singing. The telencephalic song controlling nuclei in the brain of adult male birds is 70-99% larger in spring than in fall due to the learning of new songs. Later pulse-chase experiments using ^{3}H-thymidine confirmed that, new neurons were generated from the precursors cells that were present in the ventricular zone of the forebrain[48-50].

Regeneration of retina in zebra fish is well studied, in which specialized glial cells called Muller glia (MG) proliferate and dedifferentiates into retinal cells[51]. However, in mammals such as mice, MG lack the regenerating capacity but upon injury, they proliferate and differentiate into limited number of neurons[52,53]. Recently, Yao *et al*, reported that, following gene transfer of β-catenine into mutant mice with congenital blindness restored their vision via MG proliferation and differentiation[54]. This indicates that certain genes involved in regeneration were

lost/silenced during evolution and they can be activated. In mammals the regeneration capacity is very limited to certain organs such as skin[55], blood vessels, gut, fingers[56], eye and liver. Moreover, the capacity to regenerate the tissues further decreases with age. Even in some organs it appears that, the regenerative capacity will remain for very brief period of time and later it disappears, for an instance, mice heart have great regenerative capacity after partial surgical resection but it is lost after 7days of birth[57]. During finger digit regeneration of mice and juvenile human[58] appearance of fate restricted progenitors[59] has been reported only for brief period of time. CNS has poor regeneration and do not spontaneously regenerate its axon after injury (further discussed in SCI section, for more information on evolutionary regeneration read the reviews Grandel 2013[44]; Popvich, 2008[45]; Andong Z, 2016[46]; Tanaka E, 2009[47]). Although, mammals have lost their dedifferentiation capacity in different tissues during evolution process Schwann cells can undergo dedifferentiation during peripheral nervous system regeneration. Dedifferentiation of Schwann cells was reported to be age dependent and its dedifferentiation capacity declined as the cells gets older[60,61].

1.3 REGENERATION OF PERIPHERAL NERVOUS SYSTEM AND CNS

The peripheral nervous system (PNS) is the component in the nervous system that contains all the nerves and ganglia that residing outside of CNS, however, the cell body may be located inside the CNS as for motor neurons. PNS main function is to connect CNS (brain and spinal cord) to the limbs and organs. The neurons in the PNS has maintained robust regenerative ability which often results in at least partial recovery of function after PNS damage whereas in this CNS has been questioned.

Upon PNS injury the distal part (i.e. farther from the neuron's cell body) of the nerve that is disconnected with cell body undergoes degeneration called Wallerian degeneration. The proximal part of the axons that are in contact with cell body may re-innervate their targets and complete regeneration can occur[62]. PNS contains specialized Schwann cells that myelinate axons, regulates ion and metabolite concentration and enrich regeneration of axons. In the CNS oligodendroglia produce myelin and astrocytes have many functions but importantly regulate ionic concentration. Schwann cells are divided into two types based on their function myelinating and non-myelinating Schwann cells. This myelination capacity of Schwann cells depends on the axons on which Schwann cells are in first contact with. Both Schwann cells and oligodendroglia produce and maintain myelin around axon that provides high-resistance and speed in axonal action potential of neuron. One of the reasons for the difference in regeneration between CNS and PNS is due to non-permissive environment generated by oligodendroglia and astrocytes in CNS whereas Schwann cells have stimulatory effect on axonal elongation in PNS. Following axotomy in PNS, the mononuclear cells from peripheral blood secretes cytokines such as interleukin-1(IL-1) and platelet-derived growth factor (PDGF), which stimulate Schwann cell proliferation and down regulates its myelin components. These activated Schwann cells forms cell aggregates near to axon and provide growth factors such as brain derived neurotrophic factor (BDNF)[63] ciliary neurotrophic factor (CNTF)[64] fibroblast growth factor (FGF)[65] and nerve growth factor(NGF)[63,66,67]. These growth factor promote

neurite outgrowth by stabilizing cytoskeleton of axons. Whereas in CNS injury, oligodendroglia upregulate myelin associated inhibitors (MAI), such as Nogo-A[68,69], myelin-associated glycoprotein (MAG)[70], oligodendrocyte myelin glycoprotein (OMgm)[71], and astrocytes secretes chondroitin sulfate proteoglycans(CSPG) that contain inhibitory molecules for axon regeneration (for detailed review on MAI see [72,73]). Pioneering work by Aguayo and his colleagues demonstrated that, when sciatic nerve grafts were transplanted into a CNS lesion, neurons can regenerate axons for a long distance across a lesion by means of peripheral nerve bridge[74-77]. During PNS injury neurons could upregulate regeneration associated genes (RAG's) such as c-Jun[78], activating transcription factor-3 (ATF-3)[79], SRY-box containing gene 11(Sox11)[80], growth-associated protein-43 (GAP-43)[81] where in CNS these genes were upregulated at modest levels compared to PNS. Over all, not only the extrinsic molecules secreted by Schwann cells, oligodendroglia and astrocytes makes the difference between PNS--CNS regeneration capacity but also neuronal intrinsic factors contribute to the poor regeneration in CNS[62].

1.4 CELL FUSION

1.4.1 Cell fusion and regeneration

Life starts with fusion between oocyte and sperm, which forms zygote and eventually the zygote generates a new organism. Cell fusion is not only involved in life formation but this biological phenomenon can be observed from zygote to adult animal in normal growth and development also during in tissue repair or regeneration process. *Caenorhadditis elegans* well studied in the cell fusion process in which one third of the somatic cells undergoes fusion during its normal development[82]. During development stage, larvae seam cells fusion results in reprogramming of the larval cells, which differentiate into adult terminal cells. This fusion process depended on AFF-1 (Anchor cell Fusion Failure -1) protein expression[83]. Cell fusion occurs not only in worms but also observed in across species, like in *Drosophila melanogaster* (Fruit fly), *Danio rerio* (zebra fish), birds, rodents and in mammals including humans. In humans, the fusion process observed in different developmental stages such as human trophoblasts fusing to form multinucleated syncytiotrophoblasts that enables implantation of embryo and the supply of nutrients between mother and fetus. Also, a series of events after fusion between mononucleated myoblasts progenitors leads to formation of multinucleated myotubes during muscle formation and repair[84] and macrophages fuse together and form multinucleated giant cells(MGC's) called osteoclasts during normal development and these osteoclasts maintain, repair functions and can remodel bone[85,86]. Upon fusion, bone marrow derived stem cells (BMDC) were reprogrammed to switch their hematopoietic lineage and attrain properties of other non-hematopoietic tissues during regeneration. This phenomenon was observed in wide range of tissues in rodents and human like neurons, hepatocytes in liver, myocytes in muscle and other tissues[87]. It's been hypothesized that somatic cells reprogramming after bone marrow derived stem cells fusion leads to the retainment of stem cell plasticity in different tissues[88].

1.4.2 Cell fusion and reprogramming

In late 80's, identification of cell-cell fusion process in different tissues during development and regeneration encouraged many scientists to find out the exact mechanism of cell fusion and this led to the development of *in-vitro* models to understand stem cell plasticity. Reprogramming of cells and differentiation by cell-cell fusion *in-vitro* were first reported using ES cells, where the bone marrow cells adopted properties of other cells after spontaneous fusion with ES cell[89,90]. These *in-vitro* studies paved a path to mechanisms by which adult cells change their fixed identities, that was considered impossible few years ago, and were named as reprogramming of cells. To date the mechanism of somatic cell reprogramming is well established, this can be done three different ways; somatic cell nuclear transfer (SCNT), cell fusion and direct reprogramming all depicted in **figure 2** below.

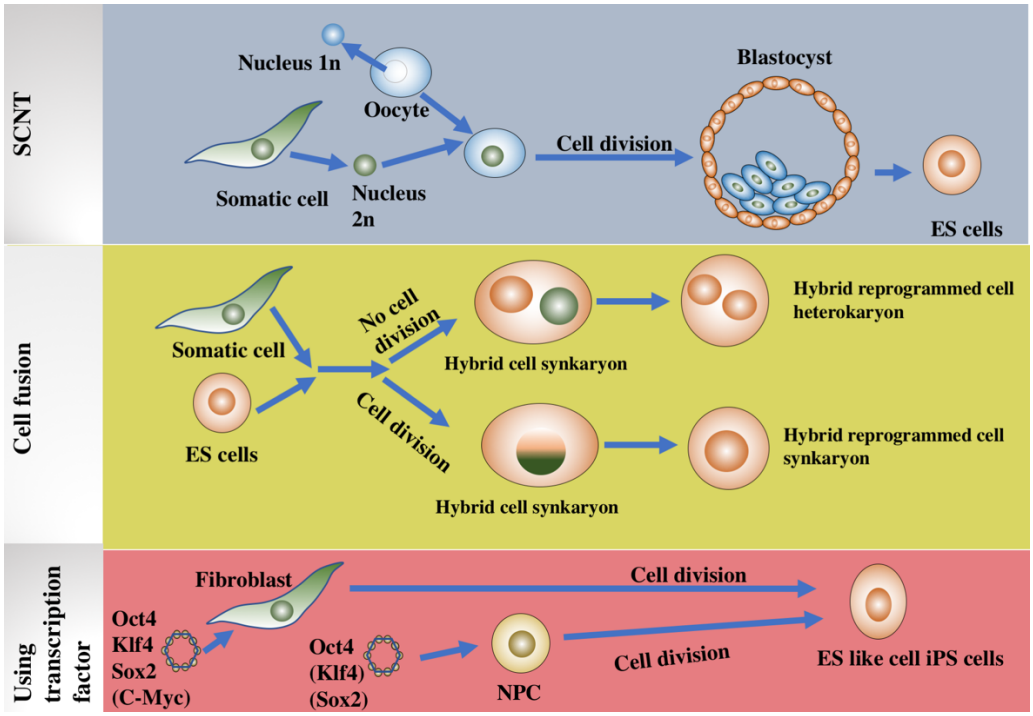

Figure 2. Nuclear reprogramming using different methods:

(Modified and reproduced with permission from springer nature; Yamanaka 2010; Dittmar, 2011[91,92])

The SCNT method aim is to generate pluripotent cells in which nucleus is transferred from somatic cells into an enucleated oocyte (**Figure 2**). This method is similar to early cloning experiment performed by Briggs and King 1952 where they generated cloned frog eggs[93], later it's been shown in sheep (Dolly) and confirmed that somatic cells can be reprogrammed to totipotent cells[94]. The major problem in SCNT is the efficiency (about 2-3%) and the survival of the clones. Early experiments in 1978 (where embryonic stem cell (ESC) cultures had not been established) embryonic carcinoma cells (ECC) were used in reprogramming somatic cells to attain pluripotency through cell fusion. Fusion experiments with ECC/ ESC and neurospheres (NSC) revealed that not only the gene expression in NSC was changed but also changed DNA, methylation, which caused NSC to lose their epigenetic memory[95]. This concluded that fusion induced hybrid cell will lose their somatic cell properties and attains

pluripotency and subsequent experiments identified Oct4, KLF as key components in reprogramming[96,97]. In 2006 Yamanaka *et al*., showed that somatic cells like fibroblast can be reprogrammed to ESC like state using Oct4, Sox2, Klf4 and C-Myc transcription factors and the cells were called induced pluripotent stem cells (iPS) (Yamanaka awarded with Nobel prize in 2012 along with John Gurdon who reprogrammed cells using frog eggs by fusion)[98]

1.4.3 Cell fusion in the CNS

Multipotent adult stem cells are defined as an immature cell that have the ability to self-renew and differentiate into mature cells in the organ they reside. The extent of regeneration of stem cells depend on their plasticity, which depends on their developmental germ layer origin. During late 1990's studies on stem cell populations using bone marrow chimeric mice ignited interest into plasticity, where bone marrow cells were labelled with LacZ or GFP that enabled their detection in non-hematopoietic cells. Upon fusion some cells expressed neuronal specific antigens like NeuN, neurofilament, and ßIII tubulin indicating that bone BMDC entered into brain and cerebellum and transdifferentiated in neurons[99,100]. Transdifferentiation of BMDC in CNS was quickly questioned, later two independent groups observed spontaneous fusion between BMDC and embryonic stem cells *in-vitro* leading to transfer of its genetic material and mixing their cytoplasm which resulted in heterokaryon formation[89,90]. This *in-vitro* experiments encouraged to evaluate this mechanism further *in-vivo*. In 2001 Nakono *et al*., transferred BMDC from male donor to female recipients so that BMDC could be identified not only with expression of GFP but also using identification of the male Y chromosome in the cells of female recipients in transgenic rodents. In these studies, bone marrow depletion was achieved either using lethal irradiation or by using the PU.1 null mouse model. PU.1 is a member of the ETS family of transcription factors expressed exclusively in cells of hematopoietic lineage. The PU.1 homozygous mutant mouse is born alive but die from severe septicemia within 2 days after birth but it can be treated with bone marrow transplant within 2days[101-103].

Human studies on post-mortem brain, from individuals who had received bone marrow transplantation due to hematological malignancies showed BMDC contributed to neuronal cells in neocortex and hippocampus[104] and a subsequent study confirmed that the fusion process takes place 1-2% neurons in hippocampus[105]. In 2003 Weimann *et al*., studied biopsies from a female who had received bone marrow from male donor and found that bone marrow derived stem cells contribute heterokaryon formation in Purkinje neurons of cerebellum. Since a sex-mismatched bone marrow was transplanted, using fluorescent in situ hybridization (FISH) technique detected tetraploid (XXXY) Purkinje neurons were detected. This led to confirmation of cell fusion between BMDC and Purkinje neurons in cerebellum. Subsequent animal studies from the same lab addressed fusion process in animal models by transferring GFP positive bone marrow donor to sex-mismatched recipients. These experiments showed Y chromosome in Purkinje neurons, further confocal images showed bi-nucleated cells indicating cell fusion. This is first clear report that provided a substantial evidence for BMDC contribution through cell fusion rather than trans-differentiation. More over the bone marrow transplantation

between rat and mouse model demonstrated that, Purkinje neurons lacked expression of hematopoietic markers (CD11b, F4/80 and CD45) and that the fused BMDC nucleus was reprogrammed to express Purkinje neuron specific genes[106]. Parabiosis experiment (a surgical union of rodents to share the blood circulation) confirmed that cell fusion between BMDC and Purkinje neurons is not due to the radiation caused by irradiation[106,107]. In 2003 using Cre/lox recombination Alvarez-Dolado confirmed the fusion between BMDC and Purkinje neurons in cerebellum[108,109]. The *in-vivo* mechanism of cell fusion is not fully understood, *in*-vitro over expression of Nanog in mouse ESC and Sall4 in mouse embryonic fibroblast resulted an increase in cellular fusion and reprogramming[110,111].

Too little is known about the cell fusion mechanism of regulation and its biological significance. Johansson *et al*. in 2008 demonstrated that the cell fusion phenomenon increased 10-100 folds during chronic inflammation such as idiopathic ulcerative dermatitis and experimental autoimmune encephalomyelitis (EAE)[109]. Hematopoietic stem cells (SP, Lin^-, $Sca1^+$, c-kit^+) could fuse with Purkinje neurons and the cell started to express Purkinje cell-specific genes (*Calb 1, Pcp 2, Kcnc 1, Gsbs*) but not hematopoietic antigens (CD45, CD11b, F4/80 and Iba1). This experiment elegantly demonstrated that, the BMDC nucleus is reprogrammed into Purkinje neuronal fate/function. These bi-nucleated Purkinje neurons were observed over long period (36-56 weeks after bone marrow transplant) which further concluded that, fusion process and heterokaryon formation are not a transient but stable processes[109].

1.5 STEM CELLS

Stem cells generates identical copies of themselves (self-renewal) and also give rise to daughter cells that have lineage-specific differentiation potentials. This mechanism of cell division where two sister cells have different fate are called asymmetric division (**Figure 3**).

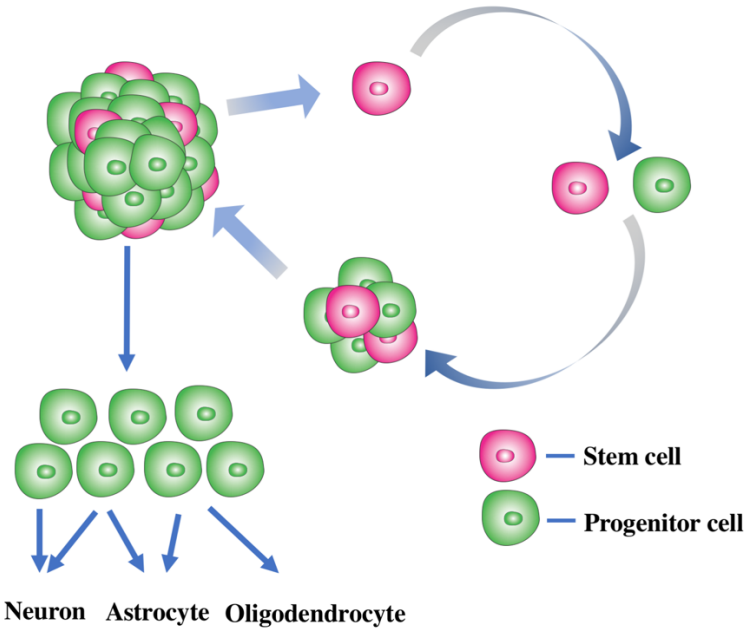

Figure 3. Illustration showing how NSC maintaining its stemness.

Several types of stem cells have been identified based on their origin and plasticity (the differentiation capacity): embryonic stem cells (ES) isolated from inner cell mass of the blastocyst, recently, reprogrammed cells called induced pluripotent stem cells (iPS cells)[98] that acts like ES cells, and adult stem cells from different tissues. Pluripotent ES cells are able to generate almost all the types of the cells in the body. Whereas, adult stem cells are differentiated and more committed or limited toward specific lineage are named multipotent. In 1981 two independent labs (Evans and Kaufman; Martin) derived ES cells from mice[112,113] and established *in-vitro* protocols for ES cells propagation, later the protocols were improved and established ES cell lines in rat [114,115] as well as in human[116]. (For ES cell historical development review see review Jun Wu, 2016[117]). However, the expression of pluripotency marker in multipotent tissues questioned the definitive term for potency[118].

1.5.1 Adult stem cells

Adult somatic stem cells or adult stem cells are rare populations of cells present in various organs and believed to be involved in maintaining tissue regeneration. These cells are multipotent, capable of self-renewal and generate progeny that differentiate into a limited number of adult cell types. Adult stem cells are usually in a quiescent state. External stimuli, such as injury, activates their proliferation and differentiation to replace the damaged tissue. There are many kinds of adult stem cells. Here, I would like to focus on adult neural stem cells[119].

Adult neurogenesis is extensively studied and it's now well established that neural stem cells reside in the adult mammalian brain in specialized niches and they contribute to tissue homeostasis[120]. Two regions were identified with large population of NSC in mammalian brain; 1. the subventricular zone (SVZ) located in the linings of lateral ventricles 2. subgranular zone (SGZ) in the dentate gyrus of hippocampus[121]. In both niches new neurons are generated and they are integrating into pre-existing neuronal circuits. NSC in the SVZ divide and generates neuroblasts that migrate rostrally into olfactory bulb where they become interneurons[122].

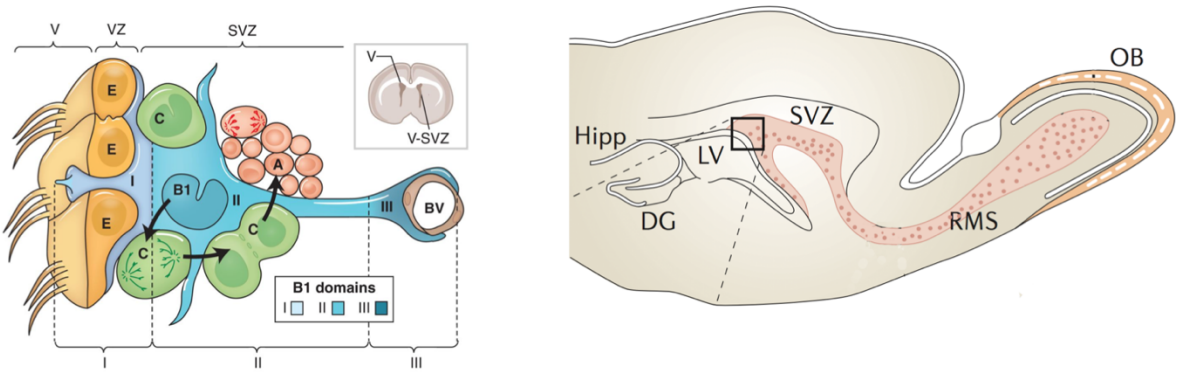

Figure 4. Illustration showing NSC migration in SVZ into olfactory bulb:

(Adopted with permission from CSHL press, Daniel *et al*., 2016)

NSC niche in SVZ has extraordinary micro-environment that facilitates cell- cell interactions and soluble factors interaction. The ependyma of lateral wall of brain composed of multiciliate cells called ependymal cells and these cells are in close contact with cerebrospinal fluid[123]. However electronic microscopic studies revealed that not only ependymal cells but type B1cells with single primary cilium were also in direct contact with CSF. These type B1 cells aggregates at the center of ependymal cell in a "pinwheel" pattern and extend a long process that in contact with blood vessel. These B1 cells are identified as astrocytes[8] in the brain expressing glial markers GFAP, GLAST and BLBP[123,124]. These B1 cells may exits the quiescent state, undergoes self-renewal by asymmetric division[125-128] and then gives rise to transit-amplifying cell or type C cell[123,129,130]. Recently, Obernier *et al*., combined short term and long-term tracing experiments with retro viral injections in SVZ showed that quiescent B1 cells generates type C cells by symmetrical division[131].

Type C cells further generates neuroblast or type A cells and expresses transcription factor *Ascl1* or *Mash1* and *Dlx2*[123]. These neuroblast expresses marker PSA-NCAM and doublecortin (DCX) and migrates in rostral migratory stream (RMS) and upon reaching olfactory bulb neuroblasts differentiates into interneurons[132] in rodents (**Figure 4**). Although the mechanism by which type B1 cells are maintaining the stem cell niche is well studies the precise surface antigens have not yet identified. Activated NSC express epidermal growth factor receptor (EGFR)[133] and recently VCAM1 expression was observed in the apical process of NSC[134]. These NSC can be readily propagated as neurospheres in presence of mitogens such as epidermal growth factor (EGF) and fibroblast growth factor (FGF).

1.6 STEM CELL TRANSPLANTATIONS

As discussed previously, CNS has poor regeneration and fewer number of stem cells in higher animals (**Figure-1**) and development of protocols that expand stem cells without affecting their potency *in-vitro* encouraged many scientists to transplant stem cells in different disease models aiming to replace cells that were lost due to injury or degeneration. There were numerous attempts made using different types of stem cell in animal models as well as in human[135]. Stem cells were transplanted in neurodegenerative disease like amyotrophic lateral sclerosis (ALS)[136], Parkinson's disease (PD)[137,138], Alzheimer's disease (AD)[139] and Huntington's disease[140] and in injuries SCI, TBI, and stroke. Indeed, studies in rodent Parkinson's model have showed transplantation of dopaminergic neurons elicit functional recovery[141]. However, in human clinical trials with 800 patients observed mixed results due to many reasons[142]. In animal model of SCI different types of stem cells were transplanted, such as mesenchymal stem cells, embryonic stem cell derived NSC, adult tissue derived NSC, human fetal stem cells, induced pluripotent stem cell derived NSC. All these studies concluded that, modest to significant recovery could be achieved in animal models. The main beneficial mechanisms proposed are mesenchymal stem cells secretion of trophic factors (NGF, BDNF, GDNF, CNTF, EGF, VEGF-A); NSC enhance axonal regrowth and promote remyelination[143,144]; differentiation of NSC into astrocytes oligodendrocytes and neurons[145-148]; NSC transplanted acute and subacute also secreted neurotrophic factors and could differentiate[149]; and also

reduced inflammation[150-152]. In 2012 Lu *et al*., demonstrated that transplanted human fetal spinal cord derived neural stem cells in growth factor matrix could extends axons across the injury[147], these results were re-assessed and confirmed[153]. This was the first evidence showing that transplanted NSC can extend their axons across the injury for about 3cm distance in rat SCI model. Similar results were obtained using human iPS cell derived NSC in rat SCI model[154]. The first phase I study using NSC in chronic spinal cord injury was initiated[155].

1.7 REGENERATION OF CNS AFTER INJURY

Our body generates new cells continuously during the process of replacing the cells that were lost due to age, DNA damage, or other processes. The similar mechanisms are engaged during replacement of cells that were lost due to injury. However, in mammals, the CNS has, in comparison to other species, minimal regenerative capacity and fails to subsequently to injuries such as spinal cord injury (SCI) and traumatic brain injury (TBI). Traumatic CNS injuries (SCI and TBI) are the one of the leading causes of death and severe disabilities are seen in the entire world[156-159]. In lower vertebrates such as Zebrafish, the spinal cord regenerates after transection injury[42]. The cause for the low regeneration in mammals might depends on the complexity of the mammalian CNS, a complex and advanced immune system and lack of sufficient number of adult stem cells. In mammals the inflammatory response to CNS injury leads to several cascades of events which eventually inhibits the regeneration **(Figure. 1)**. The regenerative response to injury depends on severity of the injury, damaged area and degree of inflammatory response[160]. The initial phase of immune response to the injury has a beneficial role to clear dead cells but later this response itself may cause irreversible secondary damage[160,161] (mechanism of inflammation is discussed more detail in spinal cord injury section).

1.8 SPINAL CORD INJURY

Spinal cord injury (SCI) is often a sudden devastating event occurring majorly by accidents and often results in permanent loss of motor, sensory and autonomic functions from below the site of injury in complete spinal cord injuries[156,162] . The global occurrence of traumatic spinal cord injury is about 750 incidents per million affecting more younger people (global average age 38 years) affecting morbidity and quality of life[162].

1.8.1 Pathophysiology of spinal cord injury

The pathophysiology of SCI can be defined into two phases, the primary injury and the secondary injury. The primary injury is due to initial disruption of spinal cord due to mechanical force/trauma and results in loss of neurons and demyelination. Following, the primary phase a series of events such as loss of neurons, demyelination, inflammation, free radical production, excitotoxicity, glial scar and cyst formation which leads to further destruction of spinal cord leading to permanent damage. To better understand the SCI, the injury events are categorized into immediate, acute, intermediate and chronic state of SCI[163]. Within minutes up to two hours after injury, axonal disruption, necrotic death of neurons and glial cells, edema, vascular disruption and hemorrhage, ischemic reaction in many segments from the injury, and a subsequent spinal shock; all these events cause instant functional loss at

and below the site of the injury. Even though the spinal cord anatomically grossly may appears normal intact after injury the integrity of the CNS may be seriously disrupted with massive activation of microglia and increased level of pro-inflammatory cytokines have been reported[164].

During acute the phase, when the secondary injury begins (2hr to 2weeks) with further vascular disruption may lead to hemorrhage and ischemia causes loss of normal autoregulatory mechanisms and increased necrosis of neurons. Neuronal loss and cellular injury results dysregulation of intracellular $Ca2^{+}$ concentration, DNA damage causes apoptotic death[165] of neurons and large number of astrocyte and oligodendrocytes[166]. Elevated levels of reactive oxygen species (ROS) are observed until 2 weeks after injury, this causes oxidation of lipids and further cellular loss and irregular intracellular Ca2+ ion concentration which in turn may cause a negative cyclic loop of neuronal loss in spinal cord. During the acute phase disruption of blood brain barrier (BBB) causes infiltration of immune cells (T cells, neutrophils and monocytes) into CNS which contributes to an inflammatory response[167]. These series of events activate astrocytes to become reactive, in which GFAP is increased significantly and the reactive astrocytes form a gliotic scar. Stem cells from the ependymal region divide and differentiates into astrocytes[13] and start to form scar tissue. Recently, Frisen's lab showed that pericytes from blood vessels proliferates and outnumber astrocytes in scar formation in Glast-CreERxR26R-YFP mice[168]. Reactive astrocytes not only form a scar in SCI but also decreases edema, helps in maintaining ionic homeostasis and permeability of BBB and decreases infiltration of immune cells[169]. During the intermediate phase from 2 weeks to 6months of SCI, where astrocytes and pericytes contribute to the glial scar formation and its maturation. From 3 weeks post injury regenerative events appear, such as axonal sprouting in corticospinal tracks, but these events are not enough to recover from SCI[170]. The chronic injury phase persists the entire lifetime from 6 months of injury[171,172]. The glial scar continues to form and injured axons undergo Wallerian degeneration. Due to massive cell death and secondary inflammation, a cavity filled with CSF called cyst often appears at lesion site. Until to date, there are no cures for SCI, routine treatments for SCI focuses on rehabilitation to improve functional outcome and coping with the consequences of the injury. Stem cell transplantation, epidural stimulation and other methods have demonstrated some recovery in animal models but these needed to be proved in human clinical trials[173,174].

1.8.2 Animal models of spinal cord injury

Different animal species were used to study SCI (pigs, cats, dogs, non-human primates, invertebrates, rabbit, rat and mice) of which 70% of the studies were performed in rats[175]. It has been reported that thoracic spinal cord injuries were mostly commonly studied in animal models and it was observed that contusion and compression type of injuries better mimics human SCI pathology[175]. I would like to focus on historical prospect of how SCI animal models were generated over time.

Several animal SCI models were developed to the understand the basic mechanisms of injury and to evaluate different treatment methodologies, of which contusion, laceration, chemical

mediated SCI were widely used. The laceration injury model is a complete or partial incision was made on the spinal cord (complete transection, incision, and hemisecting models are among them). These models are used to study the mechanism of regeneration or inhibition of regeneration across the lacerated area[75,76]. Contusion based models are popular due to their similarities to the clinical symptoms and histology of SCI. Allen in 1911introduced the weight drop model of SCI in dogs[176]. Later these models were improved and where the spinal cord was exposed after laminectomy under anesthesia and a known weight was dropped through a vertical tube on the spinal cord[177]. Later, the weight drop method reproducibility was questioned due to the bouncing effect and other practical limitations. Initial experiments of SCI were performed on dogs and cats using weight drop method. The clip compression injuries were developed in rats since rodents spinal cords were considered to be too small for the weight drop method. During compression injuries, the spinal cord is compressed rapidly with known force and predetermined time using forceps or clips[178]. More laboratories became interested in generating animal models of SCI that allowed more control over the injury and its biochemical properties matched with human SCI. However, due to lack of commercial availability of standardized SCI instruments, the reproducibility of SCI greatly challenged. Recently, contusion-based instruments were developed (OSU device, MASCIS) and also made available commercially (IH impactor)[109] where SCI generated with predetermined force using a computer[179,180]. We used IH impactor in our experiment, where force was used to deliver impact on the spinal cord. IH impactor principle is same as the weight drop method but it avoids bouncing effect and using controlled force allow researcher to reproduce the similar impacts on the spinal cord. After the SCI, the probe connected to the impactor detects displacement of the tissue, which allow researchers to pre-eliminate the animals that were not successfully injured.

1.9 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic, neuroinflammatory, demyelinating progressive disorder in CNS affecting 2.3 million people globally[181]. In 1868 the French neurologist Jean Martin Charcot documented inflammatory lesions in brain and spinal cord of patients with intermittent neurological problems[182]. MS is more common in females than males (3:1) and onset is mostly between 20-40 years of age. Eighty percent of the patients experience recurring neurological problems that are partially recovered but often worsened over time. There are three types of MS types based on symptoms and disease progression: relapsing-remitting MS (RR-MS, most common form of the disease where symptoms disappear for a period), Primary progressive MS (PP-MS,10% of the MS patients experience a continuous worsening of their symptoms) and secondary progressive MS (SP-MS, which evolves from RR-MS after certain period a time the disease). A complex interaction between the immune system, astrocytes, microglia, oligodendrocytes and their precursors and neurons result in oligodendrocyte death, axonal demyelination and axonal loss[183]. The disease is mediated through an autoimmune T cell reaction. However, the basic cause of MS pathogenesis is not fully understood. The animal models and human blood and CSF analysis confirms the

adaptive immunity (T cells and B cells)[184] and mechanisms of innate immunity (macrophages)[185] have major roles in MS. Clinical trials with drugs that limit T-cell entry into CNS ameliorated MS symptoms such as mitoxantrone, Fingolimod and B-cell depleting antibodies halt MS lesion formation and clinical signs of the disease (Rituximab or Rituxan). MS is a complex disease not only governed by genetic factors but also with extrinsic factors such as, environment, life style , Epstein-Barr virus(EBV) infection, low vitamin D, and smoking determine the vulnerability to MS[186]. Recently, it been shown that immunological mechanisms in the lung[187] and bacteria in the gut[188,189] may contribute to the pathogenesis of MS disease, however, these reports needed further validation.

1.9.1 Experimental Autoimmune Encephalomyelitis

The major pathological features of MS are inflammation and demyelination. Three principally different animal models have been used to study MS, experimental autoimmune encephalomyelitis (EAE), virus induced chronic demyelination (Theiler's virus) and toxin induced demyelination models (cuprizone and lyso-lecithine)[190,191]

In more than 100 years EAE is one of the most intensively studied animal models to understand immunology of MS. Historically, 1885 Louis Pasteur observed sporadic cases of paralysis after injecting dried spinal cord from rabbits that were pre-infected with rabies virus. In 1930 Rivers *et al*. performed a series of experiments in rhesus monkeys in order to investigate virus from inoculum caused the paralysis Pasteur cases. He included as a control group injected uninfected rabbit brain emulsion. Surprisingly two animals developed weakness in the hind limbs. Subsequent experiment clearly established the relapsing and remitting type of neurological disease after immunization[192] and also showed that perivascular infiltrated cells destructing myelin is the cause for the symptoms. In 1947 Wolf *et al*., documented resemblance of EAE symptoms with human demyelinating disease[193]. In later experiments purified myelin proteins and adjuvant were used in the emulsion used for immunization.

2 MATERIALS AND METHODS

Even though, the methods described in the constituent papers, I would like to write the methods section in detail because in scientific articles there is restriction of number of words to use. This makes some time hard to reproduce similar method in different labs. I am writing methods thoroughly so that reader can easily access the procedures if they would like to perform them. ***In paper I*** due to technical difficulties in irradiation and ethical consideration of parabiosis experiment, animal experiments such as bone marrow transplantation and EAE (section 2.3-2.4) were performed at Stanford University School of Medicine, USA. All the animal experiments were approved by Stockholm ethical committee (Stockholm, Sweden) and the Administrative Panel on Laboratory Animal Care (APLAC, Stanford University School of Medicine and the IACUC) USA.

2.1 ANIMAL BREEDING AND GENOTYPING

Paper II and III*:* Lewis rats were inbred for at least 40 generation were using in the study and C57J mice were ordered from Jax®mice. Animals were kept at room temperature (21 ± 1 °C) with 12:12h light and dark cycle and food and water *ad libitum*. Rats were obtained from Rat Research Resource Centre (RRRC, Columbia) and these LEW-Tg(EGFP)455Rrrcc transgenic rat expresses enhanced green fluorescent protein (eGFP) under ubiquitin C promoter on chromosome 5. Genotyping was performed using the protocol from RRRC to identity the GFP^+ and wildtype (GFP negative) animals. Briefly the DNA from extracted from the ear biopsy of the littermates using RED Extract-N-AMP tissue PCR kit (Sigma). After PCR and electrophoresis animals that are carrying eGFP homozygous and heterozygous were selected as donors and eGFP non-carrier siblings wild type animals were selected as the receivers

2.2 CELL CULTURE

Paper II and III: Primary neural cells (NSC) were isolated from subventricular zone (SVZ) of adult Lewis rats that were expressing eGFP according to modified protocol from Johannsson *et al*., Briefly, Animals were euthanized using 20%CO2 and decapitated, carefully cerebrum isolated from the animal kept in +4°C, PBS. ***In paper II*** the spinal cord stem cells isolated (described in section 2.9), after dissociation of isolated spinal cord into single cells using papain, the Myelin and other debris were removed by density gradient centrifugation with 30%percoll in 1xDPBS (Sigma, P1644). The supernatant discarded along with white myelin and carefully the pellet was resuspended in NSC growth medium. ***In paper III*** SVZ carefully isolated under surgical microscope and dissociated into single cells suspension using papain (Worthington, LS003126). The cells were washed in L15 medium (Life technology, 31415086) and cultured in Dulbecco's modified Eagle's medium DMEM/F12 medium (GIBCO 31331-093) supplemented with B27 without RA (GIBCO 17504044), penicillin-Streptomycin 100U/ml (Sigma, 15140122), 20 ng/ml of epidermal growth factor (EGF, Sigma E4127), and

10ng/ml of Basic fibroblast growth factor (bFGF, R&D Systems). Growth factors were added every second day and neurospheres are passaged after 4-5 days of plating.

Differentiation of NSC: NSC were grown as neurospheres, dissociated into single cells using papain then washed with L15 medium and plated on the coverslips that were coated Poly-D-Lysine. At around 10000 cells were plated per well in 24 plate and the differentiating medium containing 1% fetal bovine serum devoid of growth factors. For neurospheres differentiation assay same protocol followed using attached spheres after second passage. In paper II the concentration of nitrite in supernatants of NSC cultures was measured by Griess reaction using Griess Reagent (Sigma-Aldrich).

2.3 BONE MARROW TRANSPLANTATION

In paper I: 10-12-week-old GFP+CD45.1C57BL/6 transgenic mice used as bone marrow donor to the wild type C57BL/6 mice. The donor mice were euthanized using 20% CO2 and after cervical dislocation, carefully skin is peeled from hind limbs and femurs, tibia and humeri were removed, all the muscles were scraped away with sharp scalpel, the bones were collected in HBSS with 2.5% Fetal calf serum in ice. Both edges of the bone were excised and bone marrow isolated by flushing HBSS with 25-gauge needle, the bone marrow suspension filtered with 70mm filter (BD-Falcon) and spun at 250g for 5 minutes. The pellet washed in ice cold HBSS with 2.5% FCS, red blood lysis/ACK buffer were used to remove RBC and fraction of the cell suspension subjected to GFP fluorescence estimation by flowcytometry. Bone marrow transplantation performed in 8 weeks old C57BL/6 mice (receivers) were lethally irradiated with 4.8Gy with doses in 3 hr. interval each. Bone marrow kept in ice briefly warmed in water bath and injected into tail vein. The receivers kept under warm light and tails were soaked in warm water at 37°C before transplant. 125ul (8x107 nucleated cells per ml) of un-fractioned bone marrow suspension injected within 3-4 hr. of irradiation. Animal health were monitored everyday till the end of the experiment.

2.4 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

In Paper I: After 4-5 weeks of bone marrow transplantation receivers were checked for its bone marrow reconstitution. Animals were sedated under isoflurane anesthesia 2-3 drops of blood collected from the tail vein into an Eppendorf tube containing 1xPBS in ice. Animals were moved back into the cage and checked for 10 minuets, if there any bleeding from the tail. The Eppendorf tubes vortexed 5 seconds and analyzed for GFP expression using flowcytometry method. Wild type animal blood served as negative control and donor GFP^+ blood as positive controls in flow cytometry gating. EAE was induced in receiver (female C57BL/6 mice) animals that were 14 weeks old as described[109,194]. Briefly, the inoculum prepared by mixing myelin oligodendrocyte glycoprotein (MOG) P35-55 was dissolved in 1x Phosphate buffered saline (PBS) and mixed with Freund's adjuvant (contains 2mg/ml heat killed Mycobacterium tuberculosis in miner oil) and injected laterally into mice. Bordetella pertussis (75ng) toxin dissolved in PBS, and injected on the day of immunization with MOG

and 48h later to all the mice intravenously. Mice were checked for their health and clinical symptoms of EAE scored on 0-5 scale[109].

In Paper II: Similar to in ***paper I***, immunization was performed in 7-8 weeks old female Dark Agouti (DA) rats. Inoculum prepared by mixing 20ug recombinant MOG prepared in house with Incomplete Freud's adjuvant (IFA) in 1xPBS. At about 200ul of inoculum injected at the tail base. Animals were checked for clinical signs of EAE and documented based on EAE 0-5 scale every day.

2.5 TRANSPLANTATION OF NEURAL STEM CELLS

Paper III: Prior to the transplantation, all the animals received 10mg/kg body weight of cyclosporine s.c once daily (Sandimmun®, Novartis) for 3 days before and 3 days after transplantation. NSC were collected from the culture and centrifuged at 300xg and collected the pellet, washed in PBS and place in ice. At 8-10 days after contusion sutures were opened under anesthesia, the spinal cord exposed after removal of adipose tissue. The spinal column is stabilized using the stereotaxic frame and small incision made on the dura to insert transplantation needle. A glass capillary pulled using pipette puller (outer diameter 120 μm, inner diameter 90 μm) is attached to the Hamilton syringe using dental gum. The Hamilton syringe system fixed to a holder on the stereotaxis frame and glass capillary carefully inserted into the epicenter 1.5 mm deep into the spinal cord and placed for 5 minutes and slowly injected NSC into the epicenter. After transplantation of the cells the capillary placed for 5minutes to alleviate the pressure is to avoid NSC oozing out from the needle and removed slowly with intermediate pauses. The animals received at around 500,000-600,000 cells (single cells and small spheres $\cong$ 40μm diameter) or 50000cells/μl of 6μl volume in two injection sites. The control group received 6μl of PBS instead of NSC. The animal group in diphtheria experiment received 100mg/kg diphtheria toxin for 3 days starting immediate after transplantation with 24h resting period between.

2.6 HARVESTING SPINAL CORDS

Paper I: Mice after EAE were sacrificed at determined times after bone marrow transplantation, mice were anesthetized with ketamine, 120mg/kg and Xylazine, 10 mg/kg i.p. animals were perfused transcardially with warm 37°C PBS followed by ice cold +4°C paraformaldehyde in PBS for 24hr, the tissue is extracted from the animals and cryoprotected in 15% sucrose solution and then in 30% sucrose solution overnight. The spinal cords were sectioned at 60μm using sliding micro tome (SM2000R; Leica), olfactory bulb into 30μm, sciatic nerve 16μm (Leica CM 3000).

Paper II and III: Rats were euthanized with 20% CO_2 and decapitated for SVZ cell extraction and for spinal cord extraction for immunohistochemistry the animals were given a lethal dose of pentobarbital i.p and perfused using peristaltic pump with warm 37°C PBS followed by ice cold +4°C paraformaldehyde, whereas in experimental animals for RNA extraction and RNA-Seq experimental animals were perfused only with ice cold 1x PBS. Spinal cords that were

subjected to gene expression analysis are placed in Eppendorf tubes and snap frozen in liquid nitrogen and stored at -80°C, and for fluorescence activated cell sorting (FACS) were placed in 1xPBS for further isolation.

2.7 CEREBROSPINAL FLUID COLLECTION AND IMMUNOASSAY

Paper III: Cerebrospinal fluid (CSF) was collected from the animals before transplantation and different time points during the experiments. After euthanization cisterna magna was punctured with safety winged IV needle connected to the 1ml syringe. The CSF aspirated from the cisterna magna into 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen and stored at -80˚C for further analysis. Cytokine and chemokines were estimated in CSF using Bio-Plex Pro Rat Cytokine 24-plex Assay kit (Bio-Rad, 10014905) using 25 μl of CSF from each animal and all samples made duplets and assayed as per manufacturer's instructions.

2.8 IMMUNOHISTOCHEMISTRY

Paper I: Spinal cord section were made 60μm thick and kept as free floating in 24 well plate in PBS with 0.01% sodium azide. After washing 1xPBS three times added primary antibody Table 1 in appropriate blocking solution, free floating sections incubated overnight at 4°C in shaker; removed the primary antibody and washed with 1xPBS for three times and added appropriate secondary antibody diluted in PBS and kept at 4°C overnight or 1hr room temperature, after washing 3 times with PBS added Hoechst or DAPI for nuclear staining.

Paper II and III: For single cell immunoassay performed after differentiation of NSC and permeabilized with 4% paraformaldehyde (PFA) for 15 minutes and washed with 1x PBS. Primary antibody added (Table 1) along the 5% normal goat or donkey serum, 0.3% Triton-x 100 (Kodak), 0.01% sodium azide (sigma, S-2002) and incubated overnight at 4°C in humidor. Whereas for neurospheres the NSC were centrifuged at 300g for 5 minutes, the pellet washed with 1xPBS and post fixed with 4% PFA and incubated overnight at 4°C in blocking solution. Spheres span 300g for 10minutes for every wash. Secondary antibody diluted in 1xPBS and added to the cover glasses and spheres in Eppendorf tube incubated at room temperature for 1hr, after washing with 1xPBS three times DAPI or Hoechst added for nuclear stain and mounted with microscopic slides with Mowiol (Sigma, 81381). The tissue sectioned 16 μm thick stored at -20°C were thawed in room temperature and marked the outline of the glass slide with paraffin pen (PAP pen) and rehydrated in 1xPBS for 15 minutes. Primary antibody (Table 1) added along with blocking solution mentioned above and incubated at 4°C over night in humidor, the slides washed with PBS three times and secondary antibody added on to the slides and incubated at room temperature for 1hr and washed the secondary antibody with PBS. The nuclei stained with DAPI or Hoechst and glass slides mounted in Mowiol to protect from photobleaching.

Antibody	Dilution	Host	Manufacture	Cat.No
Anti GFP	1:1000	Rabbit	Molecular probes	G1544
Anti Calbindin	1:1000	Mouse	Sigma	C9848
Anti NeuN	1:500	Mouse	Millipore	MAB377
Anti Choline acetyltransferase (ChAT)	1:100	Goat	Millipore	AB144P
Anti beta-III tubulin	1:1000	Mouse	Millipore	MAB1637
Anti-microtubule-associated protein 2 (Map2)	1:500	Rabbit	Millipore	AB5622
Anti GFP	1:1000	Goat	Rockland	600-101-215
GFAP	1:1000	Rabbit	Dako	G9269
Gal C	1:100	Mouse	Millipore	AB142
BIII tubuline	1:100	Mouse	Millipore	MAB5564
CD11b	1:100	Mouse	Millipore	CBL1512F
Sox2	1:100	Rabbit	Millipore	AB5603
BrdU	1:100	Mouse	Amersham	RPN202
BrdU	1:50	Rat	AbD Serotec	MCA2060
CC1	1:250	Goat	calbindin	OP80
GFAP	1:500	Mouse	Dako	Z0334
IBA1	1:500	Mouse	Wako	019-19741
Olig2	1:250	Rabbit	Abcam	Ab109186
Pax6	1:500	Mouse	DSHB	AB 528427
Nestin	1:250	Rabbit	Chemicon	MAB353
GFP	1:500	Rabbit	Millipore	AB3080
GFP	1:1000	Chicken	Abcam	AB 13970

Table: List of antibodies used in the papers

2.9 ISOLATION OF TRANSPLANTED NSC

Paper III: NSC were isolated from naïve (uninjured but received NSC) and SCI animals for total transcriptome analysis RNA-Seq and NSC were sorted using Fluorescent activated cell sorting (FACS) based on the eGFP expression of the cells. Spinal cords were dissected from the animals that were perfused with ice cold 1xPBS and dissociated into single cells using 10U/ml papain (Worthington, L5003126). Myelin and other debris were removed using gradient centrifugation with 30%percoll in 1xDPBS (Sigma, P1644). The supernatant discarded along with white myelin and carefully the pellet was resuspended in 2ml of FACS buffer (1% BSA (Sigma, A8412), 2mM EDTA (Gibco ®, 15575-038), 25mM HEPES (Sigma, H0887)). The cell suspension filter using pre-wet 100μm filter with FACS buffer, and then with 40μm cell strainer. eGFP positive and negative gating for sorting arranged using eGFP positive NSC and eGFP negative wild type NSC from cell culture in BD Influx™ cell sorter.

At around 20,000 to 25,000 cells per animal were collected independently into separate tubes, the cells were centrifuged at 300g for 5 minutes and re-suspended in 1ml Trizol reagent (Thermo Fisher, 15596026), vortexed and stored at -80°C for 24hr. cells from Naïve, SCI and *in-vitro* cells were collected at 3week's and 4 weeks' time point.

2.10 RNA ISOLATION AND CLEAN-UP

Paper III: Total RNA was isolated from spinal cord tissue and also FACS cells. RNease mini kit (Qiagen, 74104) used for total RNA extraction from whole spinal cord as per manufacture instructions. Briefly, at about 15mg of spinal cord tissue weighed from each animal and stored in separate Eppendorf tube, the tissue was lysed mechanically with tissue lyser (Qiagen) in lysis buffer (Qiagen). Genomic DNA contamination eliminated using DNase (DNase I Qiagen, 79254) and RNA from isolated NSC extracted using Trizol reagent (Thermo Fisher, 15596026). Isopropanol precipitation was performed overnight using 75μg glycogen (Invitrogen, AM9516). For RNA-Seq RNA cleanup performed using RNeasy micro kit (Qiagen, 74004) according to manufacture protocol. All the sample were depleted of genomic DNA after clean-up process, the quality and quantity of RNA measured using NanoDrop (Thermo Fisher) and RNA stored at -80°C until further analysis.

2.11 RT-PCR

Paper II and III: the forward and reverse primer were designed using primer3 software and blasted sequences were verified. A total of 100 ng of RNA used to perform reverse transcription using iscript™ cDNA Synthesis Kit (Bio-Rad, 1708891). RT-qPCR was performed in 10μl reaction with iQTM SYBR® Green Supermix (Bio-Rad, 170-8887), 5μM forward and reverse primers (***Paper II and III***) using purified cDNA as template. A CFX384 TouchTM Real-Time PCR-detection System (Bio-Rad, 1855485) used for amplification (protocol used 95°C for 3 min, 95°C for 10s, 60°C for 30 s, and 72°C for 30s repeated 39 times). Two technical replicates were used for each biological replicate, plate setup, normalization of expression was performed in Bio-Rad CFX manager using ΔΔCt method, whereas in ***paper II*** the iQ v2.0 software (Bio-Rad) used for analysis.

2.12 SEQUENCING RNA FROM ISOLATED NSC

Paper III*:* Sequencing libraries for RNA from isolated NSC after 3 and 4 week transplantation were prepared using SMARTer Stranded Total RNA-Seq Kit- Pico Input Mammalian (Clontech) from total RNA. Libraries werr sequened 2x125bp on 2 lanes using HiSeq2500 (Illumina Inc) on high-output mode. For each replicate a minimum of (188e6*2)/28=13.4e6 read-pairs were used. TrimGalore (Babraham Bioinformatics) was used for removal of low- quality regions and adapter sequences. Read-pairs were aligned to a rat reference genome (Rnor_6.0) using STAR. Differential gene expression analysis was conducted in R (version 3.4.4) using packages LIMMA and edgeR (https://bioconductor.org/). Functional analysis conducted using WEB-based gene SeT

AnaLysis Toolkit (http://www.webgestalt.org/option.php/) and Uniport (https://www.uniport.org).

2.13 TRANSFECTION OF NSC

Paper III*:* NSC were selectively ablated using Diphtheria toxin receptor (DTR) expression system. The DTR system is non-integrating, self-replicating enhanced episomal vector system (EEV) which was modified (EEV600A-1, System Biosciences) used to transfect the NSC. The entire DTR-2a-mCherry flanked by restriction enzyme sequences BamHI and Xhol was synthetized at Eurofins Genomics[195,196]. The sequence was directionally cloned into the multiple cloning site of the EEV downstream of the existing CAG promoter and transfection efficiency determined by visualizing the mCherry fluorescence under microscope. EEV plasmid was purified using the EndoFree Plasmid Maxi Kit (Qiagen, 12362), and NSC were transfected using Neon® transfection system. At around 200,000 cells were re-suspended in buffer T and mixed with 0.5-3 μg DNA and electroporation set at 990V, for 40 ms, 1 pulse. The cells were immediately transferred to culture dishes containing warm NSC medium that was used for transplantation after 48hr of culture. The transfection efficiency was checked under microscope prior to transplantation.

2.14 SPINAL CORD INJURY

We established contusion model of injury in our lab, where we used commercially available IH impactor (Infinite Horizon, IH-0400). We observed that stabilization of spinal cord was a problem in our hands and that might have effect on the outcome. We modified the instruments to stabilize the spinal cord without affecting the functionality of the instrument. For this, we replaced the spinal cord holders including animal platform with a stereotactic frame that contains bilateral fixators (Kopf ® 900&900-C). We evaluated the impactor performance by confirmed reproducibility.

Paper III*:* Female Lewis rats that were non-carriers of eGFP were transported to animal surgery room one day prior to the spinal cord injury so that they adopt to the environment. All the animals were anaesthetized with 4% isoflurane (Baxter) initial induction and later to 2-3% during the operation procedure and administered 0.05mg/kg buprenofin s.c (Temgesic®, Indivior 0.3 mg/ml), 5 mg/kg karprofen s.c (Rimadyl® vet. Orion Pharma Animal Health, 50 mg/ml) animals were weighed. The fur is shaved on the back of the animal with hair trimmer (Aesculap®, ISIS 273278), applied disinfectant iodine solution on the skin and animal placed on the heating pad (37° C) and applied ointment(Oculentum simplex, APL) on eyes to stop drying. A longitudinal incision made on the dorsal side using scalpel above the thoracic spine, the muscles carefully separated without any bruise and spinal column is stabilized using the bilateral fixators in a stereotaxic frame (Kopf ® 900&900-C). Laminectomy was performed using the pincer and a surgical drill (ANSPACH ®, EMAX ® 2) at Th11, dura and spinal cord were exposed. The rat gently moved under the impactor tip (Infinite Horizon, IH-0400) and a force of 200kdyn contusion SCI was delivered. The impact was observed under surgical microscope and contusion was considered successful if animal showed an extension of the

hindlimbs followed by a flaccid palsy and the software indicates displacement of 1.5 ± 200mm of displacement and also paraplegia for two days. In the sham laminectomy group animals underwent surgery except for the contusion. The injury was covered with a piece of subcutaneous adipose tissue from the same animal in order to fill the cavity generated due to laminectomy. All the animals received basic care that include administration of Buprenorfin and Karprofen daily for three days and urinary bladder emptied twice a day until the animals recovered their bladder function.

2.15 KINEMATICS INSTRUMENT AND ANALYSIS

Paper III: To evaluate hind limb locomotor improvement by unbiased scoring system we used kinematic along with traditional scoring system described in[197]. We custom built the instrument in our lab using 100cm Plexiglas as runway platform for animals, mirrors that provide side and bottom view of the animal and videos recorded using a camera (Canon EOS 6D), camera is connected to the computer to record video (EOS utility software). In order to record animal walking patterns in every frame in detail we set camera at 60 fps an 1/800 of shutter speed. LED light is passed through the glass platform so that animal foot prints that were generated by distracting the light (**Figure 5**).

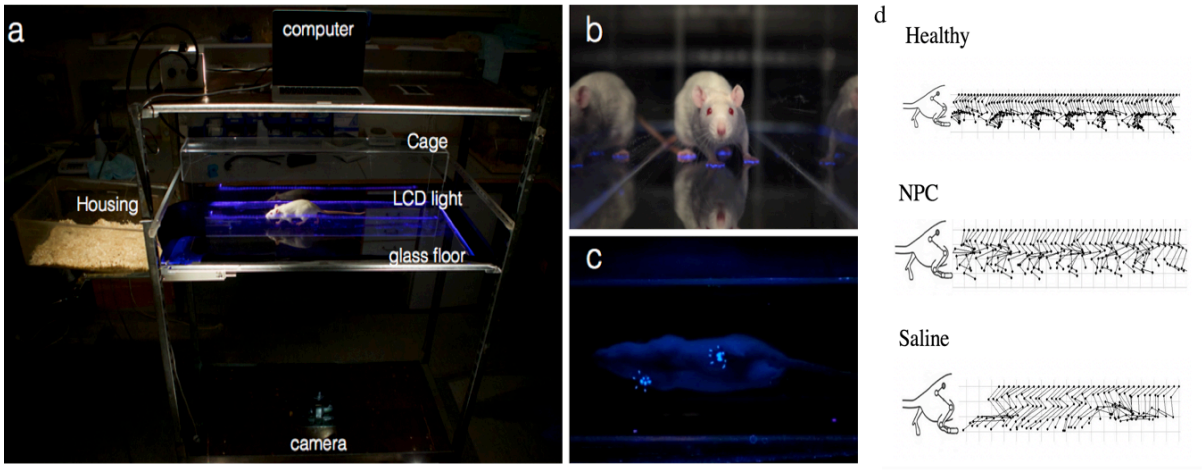

Figure 5. Kinematic instrument setup: a. custom built instrument using Plexiglas, mirrors and camera. **b-c** animal walking platform and bottom view of the animal foot prints, **d.** showing tracking of hindlimb movement from top to bottom: in healthy, spinal cord injured animals that received NSC and control animals with spinal cord injury no stem cells but saline.

To increase intensity and better visualization of footstep we prewet the glass surface. The videos were analyzed using Click Joint, AEA solutions (We tested and improved software so that it can be used in our setup). Rats fur is shaved and marked joint, Hind limb joints iliac crest, greater trochanter (hip), lateral malleolus (ankle), metatarsophalangeal joint of fifth toe, tip of the toe for tracking in the software[198]. For analysis we excluded first and last 10 cm of runway from the video only 80cm walking distance were analyzed. For paraplegic animals random frames were selected for analysis and we measured iliac crest height, stride width and

length, footsteps, velocity, protraction and retraction distance in walking. During grid walking analysis we trained the animals on ladder with constant width and while in experiment we removed random rugs from the ladder.

2.16 TISSUE CLEARING

To better understand how native and transplanted cells interact with tissue they reside it is important to visualize them in thick tissue. Traditional process like tissue section and immunohistochemistry are very good tools for this purpose. However, sectioning of tissue makes researcher to oversight crucial information where three-dimension visualization is needed. A major problem is to make thick tissue transparent without affecting its integrity and structure.

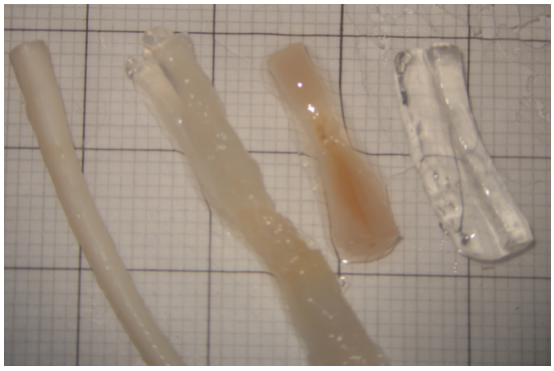

Figure 6. Optical clearing of rat spinal cord from left to right: perfused with intact myelin, semitransparent using CLARITY, See DB for 2 days and transparent spinal cord after CLARITY and see DB protocol.

Recently, number of methods are developed for this purpose. We tested and adopted different methods like CLARITY[199], see DB[200] and CUBIC methods in our lab **(Figure 6).** ***Paper III:*** For thick tissue whole spinal cord is cleared for light sheet microscope imaging optical clearing methods CUBIC-1 were used as described in Etsuo a susaki et.al[201,202]. Propidium iodide (PI) was used for nuclear staining for light sheet microscopy imaging due to its high penetration into the thick tissue.

3 AIMS

The general aim of the thesis is to investigate alternative regenerative mechanisms governed by stem cells from bone marrow, spinal cord and brain in disease models like MS and SCI.

Specific aims:

1. To investigate whether cell fusion phenomenon could occur in the inflamed spinal cord?
2. To determine effects of distant inflammation on endogenous NSC in uninflamed parts of the spinal cord.

3. To investigate if/how the transplantation of adult neural stem cells may improve hindlimb function in rat model of spinal cord injury.

4 RESULTS AND DISCUSSION

4.1 PAPER I: CELL FUSION BETWEEN SPINAL MOTOR NEURONS AND BMDC

Cell fusion process is well documented in CNS especially in cerebellum where binucleated Purkinje neurons have been identified[106,109,203-207]. We have previously demonstrated that bone marrow derived cells (BMDC's) can fuse with Purkinje cells in cerebellum and during inflammation and fusion events increased by 100 folds[109]. When other groups were analyzed the cerebellum from the multiple sclerosis (MS) patients they not only detected binucleated Purkinje cells[203,204] but also that the number of heterokaryons were increased significantly during EAE disease[203]. Even though, the exact mechanism is yet to be identified, the question remains unanswered, **is the fusion process limited to cerebellum or did it also exist in other parts of CNS such as spinal cord?**

Since the BMDC's fuses with wide varieties of the tissues like cardiomyocytes, hepatocytes, skeletal muscles, pancreas, lung epithelial tissues, kidney, skin, Purkinje neurons and other tissues during normal as well as tissue repair process[108,208-215]. **We hypothesized that 1. The cell fusion event between BMDC and CNS might be not restricted to cerebellum and might be extended beyond cerebellum**, **2. Did the rate of fusion events vary with in the cerebellum?** specifically in MS where inflammation is key player in disease progression. We used similar animal models that were used previously like EAE and bone marrow transplantation[106,109] where spinal cord was mostly inflamed. We performed bone marrow transplantation between GFP+CD45.1C57BL/6 transgenic mice as donor and C57BL/6 mice wild type mice as receiver and induced EAE after bone marrow had reconstituted fully. All the BMDC's that had entered into CNS will be expressing GFP (GFP^+) and the immune cell infiltration can be easily assessed by detection of GPF expression since, cell fusion between BMDC's and Purkinje cells results expression of GFP protein in Purkinje cells[109]. The number of GFP^+ heterokaryons were quantified in the entire neuroaxis olfactory bulb, retina, cerebrum, cerebellum, brain stem and spinal cord. To confirm if BMDC's generated heterokaryons in cerebellum, we quantified the number of heterokaryons between immunized and non - immunized mice. The number of heterokaryons were significantly more in the area where there was immune cell infiltration due to ongoing EAE inflammation. Purkinje neurons were quantified in vermis and lateral hemispheres of the cerebellum and we found more heterokaryons in the vermis as compared to lateral hemispheres. The difference in number of heterokaryons might be due to more infiltration of cells in vermis than lateral hemispheres and also, we believe this might be due to differences in the development and degeneration process within cerebellum. We also identified heterokaryon formation in the spinal cord motor neurons and some of them were bi-nucleated. The motor neuron whose cell body is located in ventral spinal cord and extends its axon into sciatic nerve and connect to muscle fibers in the hind limbs. Based on anatomical location (ventral horn of the spinal cord) and expression of GFP in nerve fiber/axon in sciatic nerve and expression of NeuN and ChAT in these cells were

confirmed to be motor neurons (**Figure 7**). We recognized some interneurons in spinal cord also capable of fusing with BMDC's and forming heterokaryons. We also detected very few MAP2^{+} neurons in olfactory bulb and in pons area of brain stem. In fact, we didn't detect any heterokaryons in cerebrum and hippocampus, this might be due to poor infiltration of leucocytes/lack of inflammation in these areas in this animal model. The absence of binucleated cells in some of the neurons might be due to 1. Technical difficulties such as, missing of complete cell body due to sectioning, 2. Transdifferentiation of BMDC's into neurons, which is least likely scenario due to complexity of motor neurons but can't be ruled out completely, 3. Recent reports on microRNA and mRNA transfer through extracellular vesicles called exosomes to neurons[216]. 4. Nanotubes transfer of genetic material; outside the CNS, mitochondrial transfer through nanotubes from healthy to damaged tissue has been reported, we might expect similar mechanisms as well[217].

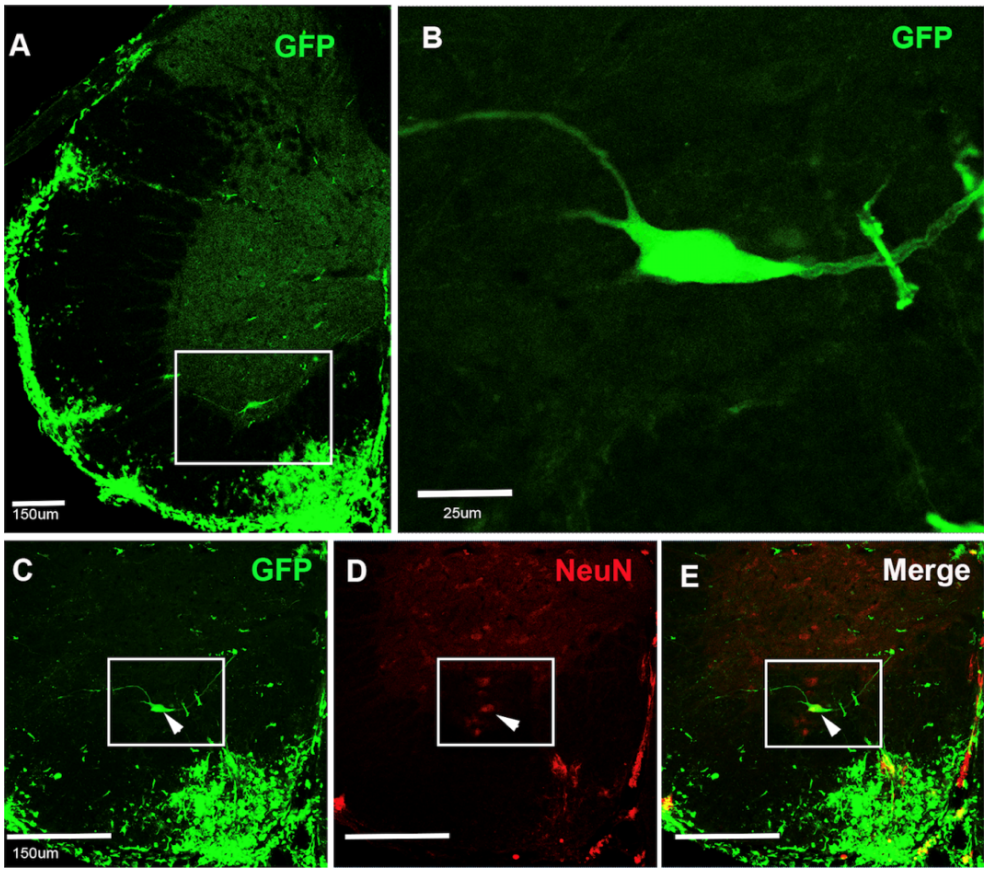

Figure 7: Cell fusion between BMDC's and Motor neuron in spinal cord: (A) Spinal cord showing infiltration of GFP^{+} leucocytes (green) and ventral horn motor neuron shown (in side box). (B-D) Motor neuron (arrow head) expressing NeuN (red) and showing colocalization with GFP (green).

4.2 PAPER II: LONG DISTANCE EFFECTS OF INFLAMMATION ON NSC

In **paper II** we addressed how inflammation affects neural stem cells niche in spinal cord where there is negligible number of infiltrated cells and the spinal cord appear normal during EAE. Lesions in this model of MS where there is active inflammation is ongoing is well documented. Non-lesion areas in CNS are great importance in understanding MS disease and progression. It been reported that when CNS from MS patients analyzed outside lesion area which appear to be normal (normal-appearing white matter (NAWM)) may contains pre-active lesions[218-221]. Altered water diffusion properties, gene expression profile and hypoxia[222]. Clusters of microglia have been reported in NAWM compared to healthy tissue which indicates that an endogenous inflammatory reaction might exist in the entire white matter of MS brain[219,220,223]. NAWM is very interesting region that might have clues in the initiation of MS lesions.

Apparently, the immediate question comes **whether the neural stem cells in the NAWM were affected by distant inflammatory dediators?** We used here EAE animal model to address this question and estimated the nitrite concentration in cell culture. The NSC proliferation and differentiation potential was compared between NSC from lesion area and NAWM area. Previously our group showed the altered gene expression and differentiation in NSC that were isolated from different regions of spinal cord during EAE[224]. Nitric Oxide (NO) is produced at elevated levels during inflammation[225]. An induced form of nitric oxide synthase (iNOS) enzyme secreted by astrocytes and macrophages is activated during inflammation[226]. Nitrite or nitrate which are the oxidized products of NO can be measured in cerebrospinal fluid of patients with MS[225,227-229] and other inflammatory diseases such as rheumatoid arthritis[230]. Since the nitrite levels are directly proportional to the degree of inflammation in MS and EAE we used this method to distinguish between inflamed spinal cord (ISC) and NAWM in NSC culture system. NSC from spinal cords were isolated, where the spinal cords were divided into cervical, thoracic and caudal parts in healthy as well as EAE rats[224] and nitrite levels in NSC culture supernatant were measured using Griess assay[231].

NSC proliferation was estimated using BrdU and it appear that thoracic NASC derived NSC have significantly higher rate of proliferation compared to NSC from healthy animals indicating that the NSC at NAWM are also affected due to inflammation. In response to injury NSC present at ependymal region proliferates, migrates and differentiates during regeneration event in spinal cord[10,13,232-234] in EAE[235], root hypoglossal root avulsion injury[11] and in stroke[236]. Gene expression analysis performed at 0hours, 24hours and 5days after differentiation using RT-qPCR method, we found that *Notch-1* gene is upregulated in both undifferentiated and differentiated culture at zero hours and later it was down regulated after 5 days of differentiation. Surprisingly the *Hes-1* (a downstream mediator of *Notch-1* signaling) expression was not matched with Notch-1 expression (0hours) indicating that Notch signaling acting through Hes independent pathway. The expression of *Mash-1* increased at 0hours in NASC cultures then, it was downregulated at 24hours of differentiation, *neurogenin 2* another regulator of neurogenesis, is also upregulated 24hours after differentiation in NASC cultures. In agreement with the pro-neural gene expression, we observed *β-III-tubulin* expression

24hours after differentiation in NASC cultures. Furthermore, immunohistochemistry quantification data (using *β-III-tubulin,* GFAP and Gal-C labelling) revealed a significant increase in neurogenesis in differentiated NSC culture from NASC than control, whereas oligodendrocytes percentage was decreased in NASC-derived cultures. This data contradicts with the previous results, where *in-vivo* setting NSC from spinal cord during EAE were differentiated in neurons[235] and NSC cultures from spinal cord and SVZ were differentiated predominantly into oligodendrocytes[237-240].Transplantation animal models affirm that spinal cord is more gliogenic than brain, however, inflammation such as EAE could reverse this in NSC.

Taken all together, this study demonstrates that 1. NSC are affected in EAE even though they were localized in non-lesion NASC area, 2. Inflammation such as in MS and EAE animal model can alter the NSC differentiation potential towards neurogenic fate and reduces oligodendrocyte differentiation. It's exciting and vital to know how inflammatory mediators produced at lesions can affect over long distance, one obvious explanation is through cytokines/chemokines that were mediated by CSF. Further studies are needed to conclude how NSC alter their fate while they were exposed to inflammation that might give insight into how NASC might develop lesions as disease progress.

4.3 PAPER III: SPINAL CORD INJURY AND ADULT NEURAL STEM CELLS TRANSPLANTATIONS

Previously, our research group demonstrated that, adult neural stem cells from ependymal region proliferates, differentiates and migrates towards the injury in hypoglossal nerve avulsion[11]. As discussed in "Adult stem cells" section SVZ are the good source of stem cells in rodents, our group also shown that upon transplantation of adult neural stem cells from subventricular zone (SVZ) differentiates into neurons and able to form synaptic connections with the host tissue after hypoglossal injury in rats[241].

An apparent follow-up question comes **whether the transplantation, differentiation and integration of neural stem cells in injury paradigm leads to functional recovery in these animals? How the transplanted neural stem cells interact with injury environment**? We choose spinal cord injury contusion model over hypoglossal injury and EAE because to evaluate functional improvement and the injury is localized than in EAE (model used in paper I and Paper II). Here we used inbred LEW-Tg(EGFP)455Rrrcc and sibling neural stem cells approach so that the tissue rejection is minimized[11,242-245]. Neural stem cells were isolated from SVZ and propagated [8,13,242,246] *in-vitro* from Lewis rats carrying eGFP under ubiquitin promoter on chromosome 5 and female rats those were non-carriers of eGFP subjected to contusion model of spinal cord injury[179,180,247-253]. After 8-10 days of post injury the neural stem cells were transplanted into the epicenter[254-257]. Several articles published in transplantation of neural stem cells and functional recovery of animals[147,149,243,245,256,258-266] in which NSCs integrates and extends axons across the injured area[147,153,267]. The major pathways in which the animals recovery is due to 1.differentiation into astrocytes, oligodendrocytes and neurons; 2. Changing the environment by secreting trophic factors like mesenchymal stem cells[268,269] and reducing

the secondary damage by interacting with microglia and macrophage[270] secreting vascular endothelial growth factor and enhancing angiogenesis[270,271]. However, these results have led to confusions in understanding the precise mechanism of action of neural stem cells in spinal cord injury models. In this study we investigated global transcriptomal changes within the NSC that were subjected to 3 and 4 weeks of transplantation in SCI. The animals that received NSC performed better in the classical BBB hind limb locomotor scaling system[272] in agreement with the previous experiments[149,243,245,256,258,262,269,273-275]. We observed the NSC had tendency to migrate and fill the cyst and differentiate predominantly into oligodendrocytes (CC1), astrocytes (GFAP) and few neurons (ß-III tubulin). The effect of NSC transplantations on the inflammatory process following SCI was analyzed using the cerebrospinal fluid from the rats that were transplanted with NSC and control animals at 3,6 and 12 weeks after SCI. We observed lower expression of the pro-inflammatory cytokines/chemokines at week 3 in NSC transplanted animals compared to saline control animals which indicates that NSC transplantation suppresses the pro-inflammatory response and might be reducing secondary damage.

To evaluate further we isolated NSC after 3 and 4 weeks following SCI and naïve transplantation (no SCI) as control animals and determined global transcriptomal changes. We found significant separation between NSC that were exposed to SCI, naïve and *in-vitro* controls which indicates NSC that exposed to injury had great impact on the their transcriptomal changes. There were many genes that control cell migration, synaptic signaling, neurotransmitter releases, axon formation, differentiation, proliferation, myelination and regulation of inflammation have been up-regulated in NSC that were exposed to SCI milieu.

Another interesting aspect of this study is apart from the classical hind limb assessment of recovery scoring system, we also performed kinematic gait analysis of hind limbs of all the rats following SCI and NSC transplantation (we custom build the apparatus explained in detail in the method section). Unbiased analysis of hind limb motor recovery is crucial to assess the effect of transplanted NSCs and more over without significant restoration of hind limb function transplantation will be futile. When we monitored the recovery using the Basso, Beattie, and Bresnahan (BBB) scaling system, the NSC transplanted animals recovered significantly better than controls after four weeks of SCI and this continued until experimental termination i.e.,15 weeks of post SCI. Iliac crest height (ICH) will be significantly lower in SCI animals[197]. We identified that ICH was higher in the SCI animal with NPC than the control rats at 6 weeks of post injury. We also performed gait analysis like step cycle, distance between the hindlimbs, all these parameters confirmed that the animals that received the NSC after SCI were recovered significantly better than the control animals. To determine coordinated stepping, we measured stepping patterns using hind limb foot prints at three, six and twelve weeks after SCI. It was confirmed that the stepping patterns in NSC transplanted animals were more similar to healthy controls. Taken all the evaluation of hindlimb parameters together it was confirmed that the animals that received NSC after SCI performed superior in recovery in hind limb locomotor function compared to the control animals.

We extended the study further to investigate the causal or side effect relationship between transplantation of NSC and enhanced recovery of hind limb function. Where we eliminated grafted NSCs immediately after transplantation and assessed inflammation profile of cytokines/chemokines in cerebrospinal fluid and hindlimb recovery using kinematics. Here we used diphtheria toxin receptor (DTR) ablation model where DTR was transfected to NSC using non-integrating self-replicating enhanced episomal vector (EEV) with mCherry (that enable us to recognize transfected NSC in red color). DTR was expressed on the surface of successfully transfected NSC undergo death by apoptosis after diphtheria toxin administration. When cells were ablated we observed that these animals had significantly lower ICH, BBB scores compared to animals with vital NSC. Moreover, we also observed elevated levels of monocyte chemoattractant protein (MCP-1) in NSC ablated animals, but this might be due to ongoing apoptotic death of NSC that attracted macrophages[276].

Finally, based on our experiments the overall possible mechanisms of recovery by which NSC exerts are 1) NSC differentiate into oligodendrocytes, astrocytes and neurons, where we observed that majority of cells differentiated into oligodendrocytes (CC1 positive) in white matter. More over gene expression analysis also confirmed the upregulation of myelination related genes, which indicates NSC in fact initiates a myelination process possibly contributing to improved function and also contributing to scar formation by differentiating into GFAP astrocytes. 2) Cytokines/chemokine analysis in CSF confirmed that NSC interacted with the injury environment and reduced pro-inflammatory cytokines which might reduce secondary damage. 3) Total transcriptome analysis identified range of genes involved in growth factor secretion, secretory molecules, and genes related to mitochondria changes. 4) Taken into consideration where we transplanted cells in the epicenter and observed NSC in entire lesion and transplantation data from immunohistochemistry and LSFM indicates NSC able to migrate in the injury even in 7days of transplantation. Since we transplanted the cells into the epicenter of the injury that will raise question that migration effect is due to transplantation itself, but as we transplanted the cell at 8-10days after injury where the cyst not yet generated due to ongoing inflammation we rule out such speculation. We believe the recovery was due to migration and transplantation the cells fill the cyst and NSCs' exerts a multi-dimensional approach during SCI that leads to hindlimb motor recovery (Illustrated in **Figure 8**).

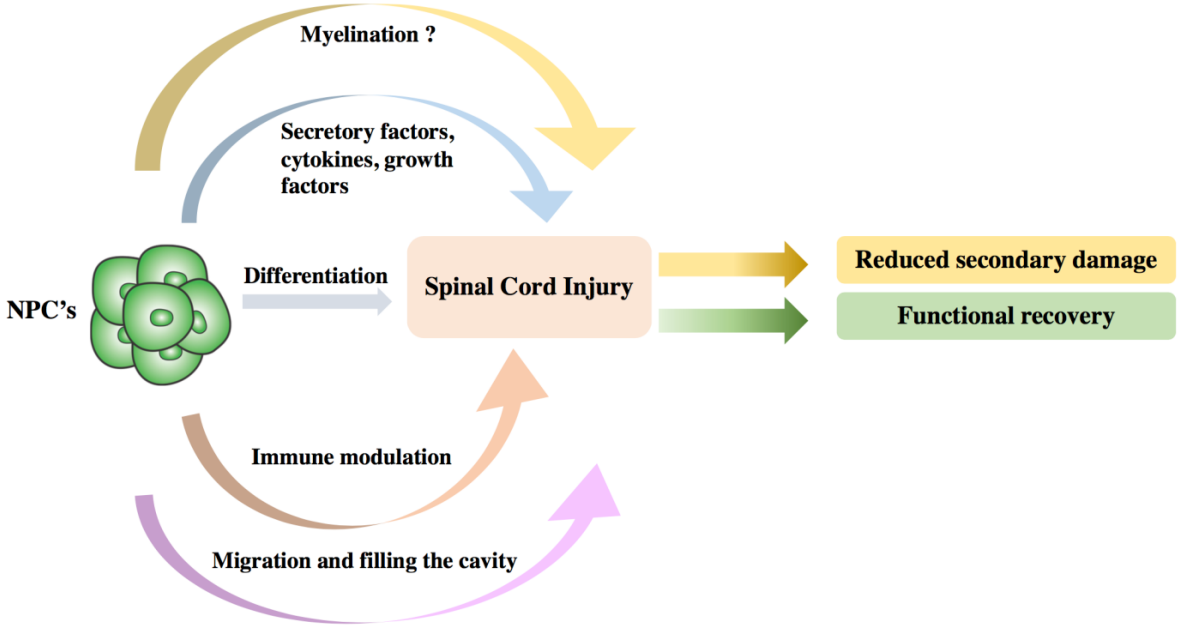

Figure 8: Overview of paper III results: Multi-dimensional action of NSC during spinal cord injury that leads to hind limb recovery.

5 CONCLUSIONS

In **Paper I.** We conclude that cell fusion is not restricted to cerebellum but it also occurs across the neuroaxis from olfactory bulb to spinal cord and it is increased in inflamed areas. We further conclude that, BMDC fuse with motor neurons in spinal cord and inter neurons during inflammation. Moreover, cell-cell fusion phenomenon increases with increased inflammation such as EAE.

In **Paper II.** We conclude that, stem cells that are distant from active inflammation were almost equally affected in proliferation and differentiation as cells in areas where inflammation was fulminant. NSC isolated from normal appearing white matter showed, altered gene expression and differentiation potential *in-vitro*.

In **Paper III**. We demonstrated that transplanted NSC in spinal cord injured rats improves their hind limb locomotor function. The majority of NSC differentiates into oligodendrocytes, astrocytes and few neurons. NSC survive in the spinal cord for 15 weeks and filled the cyst. Analysis of CSF showed NSC reduced pro-inflammatory cytokines at 3 weeks after injury and gene expression analysis indicated that the expressed genes were altered after 4 weeks of injury. Kinematic and BBB scoring system for hind limb function showed that, the animals that received NSC recovered significantly better from the injury. Diphtheria toxin induced NSC ablation after transplantations confirmed that the animal hind limb recovery was due to actions exerted by the grafted NSC.

Finally, it seems as highly differentiated neurons that are difficult to regenerate can be rescued using cell fusion while other cells possibly be regenerated by stem cell transplantation. The mechanism regulating inflammation and regeneration needs to be investigated further, which could be a stepping stone towards therapy in neuroinflammatory diseases or CNS injuries.

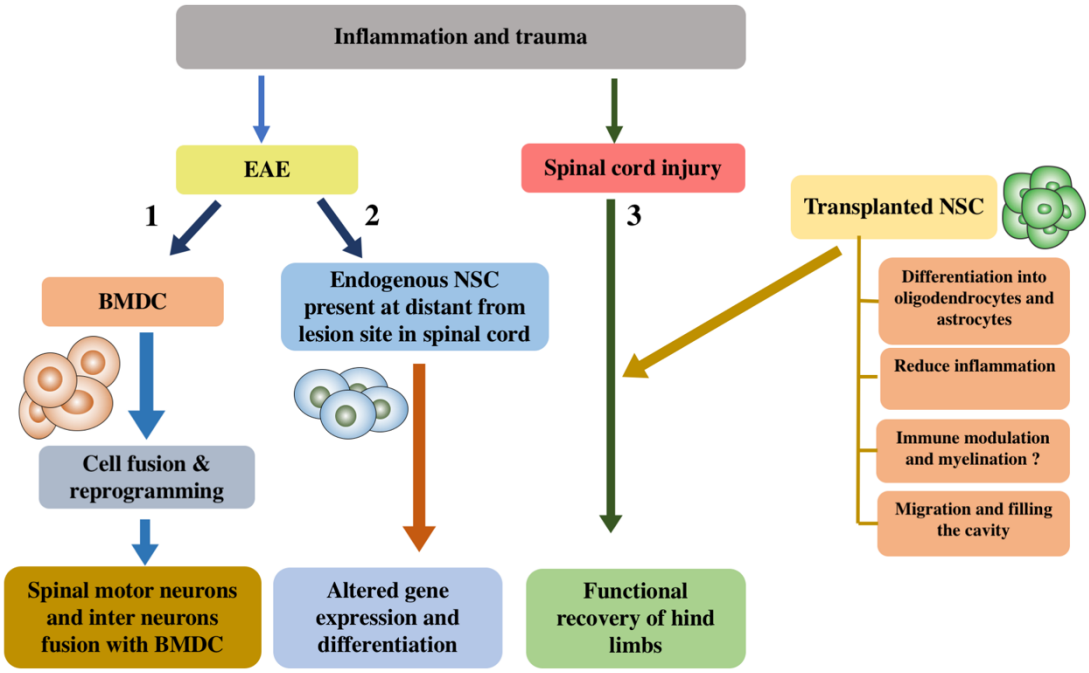

Figure 9 Illustration showing thesis conclusion: 1. Bone marrow derived cells fuses with spinal cord motor neurons, interneurons and forms heterokaryons, increase cell fusion observed during EAE inflammation. **2**. Endogenous NSC outside the lesion are affected in spinal cord during EAE inflammation. **3**. Transplanted NSC can enhance functional recovery of the animal by differentiation, migration and filling the cavity, reducing inflammation, which further reduce the secondary damage and possibly by myelination of axons.

6 FUTURE PERSPECTIVES

What is the mechanism behind cell fusion and how it used for regeneration safely?

It is very clear that most of the tissue regenerative process is associated with cell fusion phenomenon. Degeneration of Purkinje neurons were observed in many neurological disorders such as MS, spinocerebellar ataxia, stoke, murine Niemann-Pick type C1 disease and spinal motor neuron degeneration in Amyotrophic lateral sclerosis (ALS). It's been hypothesized that highly complex neurons such as Purkinje neurons and motor neurons in spinal cord, which can't be replaced with transdifferentiating approach might be protected and rescued by receiving genetic material from blood through cell fusion. Even though, cell fusion phenomenon observed in normal, pathological conditions and during regeneration and tissue repair process the exact mechanism not yet fully understood, moreover, there are several reports identified fusion process associated with tumors causes concern[277-279]. There are no reports on cell fusion induced malignancies in CNS but the precise mechanism of fusion must be identified to demonstrate clinical safety and efficacy. Identifying the precise mechanism of fusion and controlled enhancement of cell fusion events can be encouraging approach in many neurological diseases like Parkinson's and Amyotrophic lateral sclerosis (ALS). Moreover, it is necessary to identify other mechanism such as microRNA transfer by exosomes, mitochondrial exchange during fusion, transfer of mitochondria by nanotubes that were reported in CNS as well.

How NSC that are in distant from lesions in MS model affected?

The complexities of immune system that affect neurological disease makes difficult to interpret how regeneration potential of NSC changed in MS. However, it has been speculated that new lesions might arise due to inhibitory effect on oligodendrocyte progenitors in disease condition. In this study we observed that NSC in the non-affected area also changed their differentiation potentials, more over this study might give insight into the consequences of inflammation also at a seemingly unaffected spinal cord, this might be the cause of disease progression and supports the notion of early and efficient treatments

How can cytokines move around in the CNS- transferred by the glymphatic pathways?

Choroid plexus and epithelial cells act as the barriers between blood and CSF. Recently, it has been identified that, the new drainage system to eliminate soluble proteins and metabolites from CNS is called glymphatic system[280]. During MS and EAE inflammation, cytokines/chemikines are released into CSF and also NSC are identified to be in contact with CSF with their apical projection (**Figure 4** explained in adult stem cells section). There is a constant transportation of extracellular fluid and CSF allowing for transportation of cytokines and reactive oxygen species far away from their site of generation. This may occur in the CSF but also via the glymphatic system.

How transplantation of NSC leads to recovery of hindlimb function and what are the things needed to consider translating to clinic?

Initial transplantation studies with NSC from embryonic origin is a limited source, technical issues and problems with teratoma formation and may limit this type of transplantation-based cell therapy. Applying good manufacturing protocols (GMP), basic understanding of NSC made to established protocols to purify and characterize NSC, this avoiding teratoma forming cells. However, tissue rejection is major problem in transplantation therapy, even though, induced pluripotent stem cells (iPS) seems promising and are considered as renewable autologous cells in regenerative medicine, several reports have indicated immune rejection in mice experiments[281,282]. Our group previously demonstrated that, transplantation of NSC between immune compatible inbred rats resulted in integration into host tissue[241], which indicates less or no immunogenicity of grafted NSC. Here, we used similar approach, that is transplantation between immune compatible sibling rats with intact immune system, so that, the immunogenicity of grafted cells can be ignored or given least priority, while in evaluating the beneficial effects of NSC. However, the major problem in adult NSC transplantation is the source and limited availability of NSC. This needs to be solved either by using reprogramming technology or by other techniques. Even though, we and many groups observed improvement in hindlimb function in animal models SCI after NSC transplantation, it is necessary to investigate carefully the efficacy of transplantation before going to clinical trials because of substantial differences exists between humans and animals. The majority of transplantation studies have reported modest to significant recovery in animal models of SCI with different types of stem cells. This often confuse in determining which cell type is best for transplantation. Finally, the fundamental mechanisms of stem cell therapy are still not well understood and there is a need to study the basic mechanism of injury and effects of disease on the regenerative properties of the CNS.

7 ACKNOWLEDGEMENTS

Prof. Lou Brundin thank you very much for giving me an opportunity to work in your lab. I will never forget the day when you offered me a PhD position during uncertain times. You are very great person with kind heart, the way you take care of student is amazing, especially supporting me in difficult times and even tried to find accommodation. Thank you for, constant motivation, believing in me and supporting my ideas in the experiments. Your teaching skills are amazing and the way you reason the scientific question, always impresses and motivates me to do more. A simple thank you is not enough. Without your help and support I don't think I would have reached this day.

Prof. Mikael Svensson my co-supervisor, thank you for sharing your knowledge in microglia, hypoglossal injury, your pedagogic skills are impressive. You are always friendly, happy and understanding things in relaxed way. I remember during my presentations, the way you ask questions look simple but, it's very difficult to answer and you always come up with an experiment after presentation, that motivates all the students. Thank you for your help, encouragement and motivation during my PhD.

Clas Johansson, thank you for teaching me many techniques in your lab, I see a dedicated scientist in you, and I learnt a lot during my time in your lab. The way you discuss the things in lab about science kept my passion alive in science and inspired me a lot. I really wish you to continue research and all the best for that.

Maria, my co-supervisor, thank you for being friend and supervisor, thank you for your support

Rux, my unofficial supervisor, guide, friend. you're the one of the best persons I met in the lab. Lots of discussions, debates, agreements and disagreements about the projects but you are always with helping hand in science. Cell culture duties, cleaning, shoe cover, trizol so many memories I never forget. Moreover, thank you for constant supply of chewing gums and all the best for your career. Thank you very much for sharing protocols teaching transfections and for everything.

Thomas Olsson for creating neuroimmunology lab with open and innovative atmosphere and encouraging science and scientists.

Bob thank you very much for your help, advice, discussions and support. I really wished you as one of my co-supervisors. Thank you for believing and encouraging me and giving confidence.

Ingrid thank you for sharing your great knowledge in genetics, thank you for offering me an opportunity to teach tissue clearing methods in a course

Maja thank you for your discussions and valuable inputs during presentations and for sharing your knowledge in epigenetics. It impresses me how you drive the projects with great passion, ambition and determination. You are very inspirational to young scientists

Fedrik phiel, thank you for valuable scientific inputs and NHR information, life discussions, thank you for believing in me and giving me an opportunity to teach in medical course.

Anna Fogdell thank you for discussions and sharing knowledge in virus and MS

Mohsen, thank you for being good lab manager and for ordering, delivering duties, social talks especially about men age and hair, and for organizing football bets and Christmas dinners and for creating neuroimmunology a friendly atmosphere.

Andre, thank you for being kind and helpful, thank you for teaching flowcytometry, and introducing me to your T-cell world. Your honesty and passion for science is motivational to me. Thank you very much.

Leonid thank you for being my mentor and always trying to help me and motivating in PhD. You always take initiative to discuss research and your commitment as a mentor keeps me encouraged. I will be always thankful to you. thank you for your support.

Gustavo, thank you for your great inspirational words and suggestions in research. You are the doer in science with very good heart, thank you for your pizzas those you baked at your home. **Maria,** thank you for inviting to your home.

Alex, you always an inspirational scientist with your great knowledge and talks on gene editing concepts, which I am interested as well, I feel, I never wasted one second while discussing with you, every discussion I learnt something new. thank you for sharing your great knowledge.

Ramil: my friend, partner in research, thank you for joining in everyday discussion, our Thursday's philosophy discussion over lunch. You gave me lots of motivation in life. You always amaze me the way you work, you are very unique and new inspiration. We are the team BRAVO and two puppies working in the lab…☺. We should continue our collaboration 1+1=11. Thank you for being best friend.

Arvid: Thank you for being kind, jovial and helping in building the kinematic instrument, the way we were roaming in underground for recycled material I can't imagine now we did that. We are in same boat, we started PhD together and glad that we are ending at the same time. I still laugh and remember every Christmas, with your beautiful greetings card that you sent us 4 years ago. Now you are into more philosophical mood come back to jovial mood. You are such a good friend thank you and all the best for your PhD.

Sohel: My dr dr friend, you are very nice person thank you for your help during my move to Sigtuna. We had lost of discussions outside lab about life, career etc., hours of walking with debates. We haven't organized get together even after planning from 1 year. You are definitely one in my best friends All the best for your Swedish medical exam.

Tojo: Thank you for your friendship, long walks, discussions and cycling. Thank you for help in moving and for sharing housing market trends and I am happy that we are finally finishing PhD together. Thank you for sharing your PhD application process and helping me in that.

Momin: the gadget guru, thank you for your conversations during lunch and sharing your knowledge on Tesla and electronic gadgets.

Sussie my dear tall sister, We are in the same condition, you will sail through it, thank you for your help and all the best for your PhD, **Lisa** thank you for being my friend, Its always happy to discuss research with you **Nasren** thanks for nice and funny talks, you are true person with kind heart **Mikael Fagerlund** thank you for sharing your knowledge in transplantations, **Jonathan** thank you for your jokes and keeping smile on everyone face, **Johan Jacobsson** for being my student and friend, **Jonas Grippenland,** Mr.cool, thank you for being cool and fun during microRNA project, **Britt, '**My Mother in Sweden', thank you for great help during animal perfusion and lunch discussions, without you, neuro & surgery room will be unimaginable, thank you for being very good lab manager **Pernilla,** thank you for being kind, solving some administrative problems and for helping , **Caroline,** thank you for lots of discussion on aquaporins and we are for long time motivating each other, all the best for your PhD, **Sebastian,** thank you for sharing your vast knowledge and protocols on motor neuron and their differentiation, **Erik,** you are Mr. perfect, thank you for trying to help me when things are in other side, thank you for summer grill and for everything. **Per,** thank you for loud laughs in the lab and make everyone smile with your spontaneity and guitar songs, **Bomme**, thank you for nice discussions and sharing knowledge **Paula,** thank you for being good lab manager and organizing, creating lovely environment to work in the lab and thank you for your help, **Anki,** thank you for nice discussions during lunch about good clinical practices and vegetarian food. **Cynthia & Pendar,**, thank you for your support, **Christian,** thank you for getting the foot print concept to the lab, I still remember you translated glue as oil in Swedish and when I used that glue, the doors were jammed and finally we end up in changing the doors. Thank you for fun and discussion.

Brinda, thank you for your help in everything, nice talks, career, housing, Indian food, etc., lot of things thank you for all. **Lotta**, for being nice and walking from Kungshamra **Nannis**, for being nice, thank you for your lovely discussions, thank you **Mei** for being calm and **Alvin** for being nice, **Heidi** for nice discussions, housing suggestions and help **Cecilia**, thank you for being kind and honest, for inviting us to the summer party, organizing grill, it's been pleasure to talk to you always. **Lara** it was nice talking about the manuscript submission, projects, post-doc life discussion and other casual talks, thank you for friendship. Thank you, **Xingmei** for constant laughs and talks, funny discussions and your innocence, **Toni** for sharing your knowledge on injections and pain measurement in mice **Venus** for lab ordering, MOG, fancy cakes and your help in ordering and inviting us for parties. **Pernilla Stridh** thank you for your help, suggestion and support**, Patrik** thank you for happy, being kind and good discussion on career**, Fiaz** thank you for sharing antibody, protocols and for your help, **Hannes** my dear dr.dr. thank you for giving us positive controls and primers for QPCR optimization, **Eliane,** for

helping in RNAase free room and discussions, good luck with your PhD, **Jinming** and **Majid** thank you for nice discussions during parties and weekends in the lab, Thank you**, Mathias** for talks in lunch room and helping in getting a Cyclosporine A for animals**, Rasmus,** thank you for helping in choosing vegetarian food in parties and friendship, **Ewoud**, 5th floor Darwin (for beard), thank you for nice discussions, parties and friendship Thank you **Sandra**, **Daniel Ramsköld**, **Sahl**, **Jessi**, **Keying**, **Marie N**, **Galina**, **Melanie**, **Petra, Magdalena, Shahin**, for help, discussions and being nice, **Sabrina,** for opening the door during weekends when I forget the access card and thank you for your advice and help during PhD application process, **Karl**, thank you for being nice and helpful in the lab, and discussion about fatherhood, it's sad that we didn't collaborated well. All the best for your PhD. **Sevi,** thank you for your friendship, talks about career and Swedish, **Dimitris,** thank you for your conversations and friendship.

I would like to thank to my previous labs, **Jamie Morrison** thank you for allowing me to work with salamanders and confocal microscopy in your lab, this motivated me to work on regenerative medicine field, **Prof. Erik Sundström** Thank you for sharing your knowledge in SCI and stem cells during my master's degree project it helped a lot in my PhD.

Nada, thank you for nice talks and suggestions, **Roham** and **Harald**, thank you for your help and sharing your knowledge you guys are new inspiration to the lab. **Yu Li** thank you for your help and support **Hannah** thank you for your help in manuscript preparation. Thank you**, Annika,** for cell sorting work. **Ingela,** thank you for nice talks and being very good friend, **Khaled,** thank you for helping in accommodation and for being good friend.

Thank you **Senthil,** your interest in science is amazing, you always teach me something while in discussion. Thank you for your help, advice and friendship. Thank you all my fellow Indian friends **Akhilan &Vishnu Priya** thank you for being nice friends and discussion over lunch , **Ajay, Vijay Joshua** thank you for your friendship, **Shanker** you always impress me with your 1-2 years of planning in your calendar, thank you for your friendship, **Vivek & Saranya** brother you are wonderful person, you are always with "take it easy" attitude and cheerful that inspires me, thank you for being a very good friend , **Jayesh &Tina** loved your friendship thank you very much**, Nageswara Rao** brother thank you for your advice, discussions and help. Your easy-going attitude, funny conversations make you unique and thank you for your friendship, **Nagabothu, Sindhu & Sriksha,** thank you for your friendship, **Srinu N** we started masters same time and PhD in KI, happy that we are finishing it together, all the best with your PhD**, Sai Kiran** thank you for your career advice, your passion towards science really inspired me a lot. Thank you for your friendship.

Thank you **Gunilla, Marie, Romas, Jose, Jeff** for your help and support. **Mathilda**, **Nettan**, **Bettan, Josefine** and **Beser** for taking care of Vishruth

Vijay, Ashwini and Aarush thank you for being nice friends and get together and movies, travel plans, games, fun etc., **Vijay,** we came to Sweden same time I am happy that now we both settled here in same way. You guys are the true friends to me in Sweden. **Stalin** thank you

very much your help while shifting, **Nagarjuna& Sushmita** thank you for nice discussion in summer parties. **Sandeep** thank you for your friendship and conversations over phone

Thank you, AKM animal house staff, **Sandra, Velina, Björn, Linda, Mirre, Kicki, others** for taking care of the animals. Thank you CMM IT and administration always helping when there is a problem.

My family, one thing is for sure, nothing will come before family, because they are with me all the time, during good and bad times and always support and encourage me. My mother **Padma (Amma) & Father Dr. Venkata Ramana Charyulu (Nanna),** you guys sacrificed so many things for my happiness with love, care and affection. Whatever I am today it's all because of your hard work and commitment towards me made it possible, **Nanna** from childhood, sharing your medical knowledge on disease and biology generated curiosity in me to do research. Your strict discipline methods made this possible. I really miss you today. Even before your last breath, you were with your medical passion and social service (treating economically weak patients) and I hope that you will listen to this in heaven.

Brother **Veera Raghavan (Raghu)** & **Bhuvana (vadina)**, thank you very much in believing and motivating and supporting me in all the time. **Raghu**, I never forget during tough days how you took responsibilities and drive family forward, there is no doubt, without your help and support I won't be reaching this today. **Abhiram**, **Avani, Kaushik, Sravani&Ajith** for your love, affection and cute chatting. Sisters **Krishnaveni (Akka), Rama Devi (Akka)** for the fun, food, love and affection and **Ragappa (bava)**, **Venu (bava)** for help, advice and support. **Rama Chandra Chary (Mamayya)**, **Padma (Attayya)**, **Srinivas (bava)**, **Haritha** and **Shritha** for conversation over internet and keeping me happy, **mamayya** and **attayya** thank you for nice food, love, affection and hospitality towards us **Vamshi Krishna**, **Radhika**, **Sathvik**, **Samith** thank you for your love, support, help, encouragement, discussions and fun.

Sandhya, my life taken another turn after we got married. starting of PhD and our wedding happened at almost same time. Now I am completing PhD, at least from now onwards I will be spending more time without any stress with you. Thank you for being wonderful partner, understanding and adjusting to my lifestyle. So many sacrifices you did I never forget. You are always being calm and polite to me, creating positive energy in me. Thanking you for suggestions, corrections, fun, love, affection, care, food and taking care of family and trust in me. Thank you for coming into my life and helping and supporting me. **Vishruth,** my son, after you, my life has changed, with your cute smile, little things you do make me happy every second, night and day. You gave me happy memories, those changed my monotonous life into colorful joy life, I will have these for my entire life. I never forget your cute first walking steps in Kungshamra. So many memories, everyday new things, no doubt you are my 'stressbuster', motivator to learn Swedish and YOU ARE MY LIFE.

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